Impact of radiation, temperature and growth stage on the concentration of flavonoid glycosides and caffeic acid derivatives in red leaf lettuce

vorgelegt von Diplom-Biologin
Christine Becker
aus Kassel

von der Fakultät III – Prozesswissenschaften
der Technischen Universität Berlin
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Vorsitzender:	Prof. Dr. S. Drusch
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Gutachterin:	Prof. Dr. M. Schreiner
Gutachterin:	Dr. A. Krumbein

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Summary

Among the many purposes flavonoids and phenolic acids are serving in plants is protection from radiation. It influences their concentration alongside with temperature and ontogeny. Red leaf lettuce (*Lactuca sativa* L. var. *crispa* L.) contains cyanidin, quercetin, and luteolin glycosides and caffeic acid derivatives which promote human health – partly due to their antioxidant activity. In cool seasons in Central Europe, lettuce cultivation in greenhouses often consumes a lot of energy. Lowering cultivation temperature or applying energy saving (but radiation reducing) screens can improve their CO_2 balance but also influence the concentration of phenolics. In this context, the impact of low photosynthetic photon flux density (PPFD) and low cultivation temperature on lettuce in different growth stages were studied. Phenolics were analyzed via HPLC-DAD-ESI-MS^n.

Detected effects were highly structure dependent.

In a growth chamber experiment, quercetin and luteolin glycosides responded strongly to PPFD reduction (410 to 225 µmol m^{-2} s^{-1}). Yet, remarkably, temporary reduction in early growth stages did not permanently decrease the concentration of phenolics. In the greenhouse experiment, the sensitivity of flavonoid glycosides to even lower PPFD ranges (230 – 43 µmol m^{-2} s^{-1}) could be shown for the first time via multiple regression analysis. A most interesting interaction between plant age and PPFD regarding cyanidin glycoside was revealed. Younger plants' concentration of phenolics generally exceeded the older ones'. In another growth chamber experiment, cyanidin glycoside accumulated due to low temperature (12/ 7 °C compared to 20/ 15 °C at day/ night with 247 µmol m^{-2} s^{-1} PPFD), especially in young plants. Against all expectations, quercetin and luteolin did not accumulate – as long as plants in corresponding growth stages were compared. Caffeic acid derivatives were unresponsive to PPFD and mostly to low temperature. The observed differential accumulation of flavonoids might be due to different reactive oxygen species induced by the respective impact factors.

Energy saving screen application only in the first weeks of greenhouse lettuce cultivation appears feasible. Lower temperature in the remaining time until harvest may increase cyanidin glycoside concentration but postpone development of marketable lettuce heads.

Kurzfassung

Flavonoide und Phenolsäuren schützen Pflanzen unter anderem vor Strahlung, welche – neben Temperatur und Ontogenie – deren Konzentration beeinflusst. Roter Blattsalat (*Lactuca sativa* L. var. *crispa* L.) enthält Quercetin-, Luteolin- und Cyanidinglykoside und Kaffeesäurederivate mit gesundheitsfördernder Wirkung – teilweise auf Grund ihrer antioxidativen Aktivität. Salat wird in Mitteleuropa in kühlen Jahreszeiten häufig unter großem Energieverbrauch in Gewächshäusern angebaut. Niedrigere Anbautemperaturen und (leider strahlungsmindernde) Tagesenergieschirme können die CO_2-Bilanz verbessern aber auch die Phenolkonzentration beeinflussen. In diesem Rahmen wurde der Einfluss verminderter photosynthetischer Photonenflussdichte (PPFD) und niedriger Anbautemperatur auf Salat verschiedener Wachstumsstadien untersucht. Phenolische Substanzen wurden per HPLC-DAD-ESI-MS^n analysiert.

Die gefundenen Effekte waren deutlich strukturabhängig.

In einem Klimakammerversuch reagierten Quercetin- und Luteolinglykoside stark auf reduzierte PPFD (410 auf 225 µmol m^{-2} s^{-1}). Temporäre Strahlungsminderung in frühen Wachstumsstadien führte aber nicht zu permanent reduzierten Konzentrationen. Multiple Regressionsanalyse konnte im Gewächshausversuch erstmalig die hohe Sensibilität von Flavonoidglykosiden gegenüber noch geringerer PPFD-Bereiche (230 – 43 µmol m^{-2} s^{-1}) zeigen. Zusätzlich wurde zum ersten Mal eine Interaktion zwischen Pflanzenalter und PPFD bezüglich des Cyanidinglykosids demonstriert. Die Kaffeesäurederivate reagierten nicht auf die getestete PPFD. Generell übertraf die Phenolkonzentration jüngerer Pflanzen die der Älteren. In einem weiteren Klimakammerversuch akkumulierten besonders in jungen Pflanzen Cyanidinglykosid und Kaffeoyläpfelsäure infolge niedriger Temperatur (12/ 7 °C verglichen mit 20/ 15 °C tags/ nachts, bei 247 µmol m^{-2} s^{-1} PPFD). Unerwartet war dies bei Quercetin- und Luteolinglykosiden nicht der Fall, solange Pflanzen in korrespondierenden Wachstumsstadien verglichen wurden. Die differenzielle Akkumulation könnte eine Reaktion auf die Bildung unterschiedlicher reaktiver Sauerstoffspezies durch die Einflussfaktoren sein.

Es scheint machbar, Tagesenergieschirme in der ersten Anbauzeit einzusetzen. Niedrige Temperatur in der Folgezeit kann die Cyanidinglykosidkonzentration erhöhen aber die Entwicklung marktreifer Köpfe verzögern.

Experiment 1 is published in **Plant Physiology and Biochemistry:**

Title: “Temporary reduction of radiation does not permanently reduce flavonoid glycosides and phenolic acids in red lettuce.”

Becker, C.; Klaering, H.-P. Kroh, L.W.; Krumbein, A. 2013.

Plant Physiology and Biochemistry 72: 154-160.

http://dx.doi.org/10.1016/j.plaphy.2013.05.006

Experiment 3 is published in **Food Chemistry**:

Title: “Cool-cultivated red leaf lettuce accumulates cyanidin-3-O-(6‘‘-O-malonyl)-glucoside and caffeoylmalic acid.”

Becker, C.; Klaering, H.-P. Kroh, L.W.; Krumbein, A. 2014.

Food Chemistry 146: 404-411.

http://dx.doi.org/10.1016/j.foodchem.2013.09.061

Experiment 2 is published in **Journal for Agricultural and Food Chemistry**:

Title: “Unlike Quercetin Glycosides, Cyanidin Glycoside in Red Leaf Lettuce Responds More Sensitively to Increasing Low Radiation Intensity before than after Head Formation Has Started.”

Becker, C.; Klaering, M. Schreiner, H.-P. Kroh, L.W.; Krumbein, A. 2014.

DOI: 10.1021/jf404782n

Results reported in this thesis have been presented in 3 international and 6 national talks as well as in poster contributions to 2 international and 3 national conferences (see p.109-110 for details).

Table of contents

1 Introduction

1.1 Botanical and horticultural background of *Lactuca sativa* L.

Lettuce (*Lactuca sativa* L.) is an annual plant of the Asteraceae family which comprises a large number of species well-known to horticulture. Apart from lettuce, other Asteraceae vegetable crops are endive and chicory (*Cichorium endivia* and *C. intybus*), artichoke (*Cynara scolymus*), dandelion (*Taraxacum officinale*), black salsify (*Scorzonera hispanica*), and topinambour (*Helianthus tuberosa*).

Around 2600 BC, lettuce domestication started in Ancient Egypt where wild lettuce was first cultivated for its seed oil. Regular consumption of lettuce leaves has not been common before the Ancients Greeks. In Central Europe, lettuce is first mentioned in writing in 795, yet head forming types were not documented until 1543.

The vegetative phase of lettuce ontogeny[1] starts with the seedling developing into a rosette which will later form the lettuce head. The process of head formation is based on re-orientation of leaves from their primary horizontal rosette state towards a rather up-right position, progressively bending inwards to form a bud (= head). In this growth stage, there is very little shoot elongation but ample leaf growth. Once head formation is completed, the plant leaves the vegetative and enters the generative phase, visible by shoot elongation and inflorescence development.

The wide range of current *L. sativa* cultivars putatively stems from a weed called prickly lettuce (*Lactuca serriola* L.). The cultivars differ regarding the shape of their leaves as well as rosette density and the time span plants spend in the rosette stage. Most common types in Europe and North America are head, romaine, and leaf lettuce which are all cultivated for their leaves. Romaine lettuce (*L. sativa* var. *longifolia*) has long leaves which form a loose head. It is mainly cultivated in the Mediterranean. Head lettuce (*L. sativa* var. *capitata*) is divided further into butterhead and iceberg lettuce. While the mostly rich green, smooth leaves of butterhead form a relatively closed head, the light green, rougher

[1] Ontogeny comprehends the complete development cycle of one organism as distinct from phylogeny describing the history of development of species or whole phyla from an evolutionary perspective.

leaves of iceberg gather to form a very dense, closed head. Butterhead is mainly cultivated in Central Europe while iceberg is very popular in the USA. Leaf lettuce (*L. sativa* var. *crispa*) is quite a new type of lettuce whose leaves only aggregate to relatively loose heads. It comes in a color palette from green to red and a large variety of leaf shapes. Red Lollo (also known as Lollo Rosso) and red Oak Leaf lettuce are common examples of red pigmented semi-heading leaf lettuce (fig. 1). Asparagus lettuce (*L. sativa* var. a*ngustana*) is a somewhat different type and is still quite close to *L. serriola*. Its thickened shoot is commonly consumed in China and Egypt.

Figure 1: Different types and varieties of lettuce (*Lactuca sativa* L.): green butterhead (picture by Martin Sandmann) and two red leaf lettuce cultivars: red Lollo and red Oak Leaf. The two red leaf lettuce varieties were studied in this thesis.

Cultivation of romaine, head, and leaf lettuce is restricted to the vegetative phase. Rosettes that have turned into sufficiently dense and heavy heads are harvested before the shoot elongates. Some leaf lettuce cultivars are harvested before the rosette stage is abandoned and put on the market as baby leaf lettuce. In the European Union, lettuce is an important crop (Baslam et al. 2013). In Central Europe, cultivation commonly takes places in the open field in summer and in greenhouses from September to March as lettuce is adapted to warm-cool climate of the temperate zone. Over the year, producers can access a multitude of cultivars adapted to the respective day length, radiation intensity, and temperature.

Lettuce contains a number of phytonutrients – plant metabolites known to promote human health (Martin et al. 2011) – including tocopherols, ascorbic acid, riboflavin, folic acid, phylloquinone (= vitamins E, C, B_6, B_9, K), carotenoids, and minerals such as potassium, magnesium, calcium, iron and zinc, yet mostly in moderate amounts (Krug et al. 2002; Mou 2009). Apart from these compounds, especially red leaf lettuce contains polyphenols like flavonoids and caffeic acid derivatives (Llorach et al. 2008) which will be the focus of this thesis.

(Information on history and cultivation was taken from Krug et al. (2002).)

1.2 What is the function of flavonoids and phenolic acids in plants?

Flavonoids and caffeic acid derivatives are secondary plant metabolites of low molecular weight. In contrast to primary metabolites like amino acids, sugars, and fatty acids, secondary metabolites are not considered essential for plant survival. Nevertheless, flavonoids do provide key functions in plant growth and development and are essential for survival under certain circumstances: They grant enhanced tolerance to a variety of abiotic stressors, act as defense agents against herbivores and pathogens, attract animal vectors for pollination and seed dispersal, and mediate mutualistic interactions with nitrogen fixating bacteria or arbuscular mycorrhizal fungi (Gould and Lister 2006).

The class of flavonoids comprises six subgroups: flavonols, flavones, isoflavones, flavan-3-ols, flavanols and anthocyanidin (Crozier et al. 2007). Numerous substituents can be found at the basic skeleton (fig. 2), usually several hydroxyl groups which, like glycosydic sugars, increase the molecule's water solubility. The flavonoids addressed in this thesis are glycosides of quercetin (flavonol), luteolin (flavone), and cyanidin (anthocyanidin).

Although they are inducible by processes as diverse as wounding, pathogen infection, high light chilling, ozone, and nutrient deficiency, colorless flavonoids like quercetin and luteolin are best known for UV protection (Gould and Lister 2006). Yet, all of these scenarios can be united as antioxidant protection, which prompted Pollastri and Tattini (2011) to suggest this as their first and foremost function.

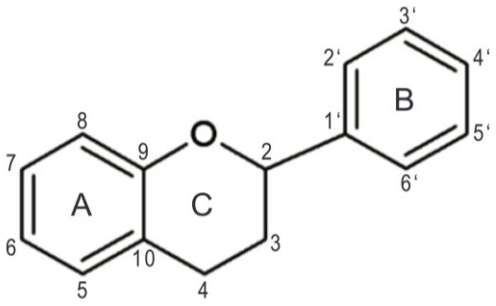

Figure 2: Flavonoid C_6-C_3-C_6 skeleton (based on Crozier et al. (2007)

An *ortho* 3',4'-dihydroxy moiety imparts great antioxidant activity (Rice-Evans et al. 1997) and can be found in the B ring of the flavonoids and the phenolic moiety of the caffeic acid derivatives studied here (fig. 3). Flavonoids outperform other well-known antioxidants like α-tocopherol or ascorbic acid (Gill and Tuteja 2010).

Colorless flavonoids indeed do absorb UV radiation and when accumulated in epidermal vacuoles they certainly shield tissue below from a proportion of these wavelengths. Yet, flavonoid pools have also been discovered in the cytoplasm of mesophyll tissue where they are unlikely to function as UV protectants (Gould and Lister 2006). Quercetin and luteolin have also been detected in chloroplasts in several species which puts them in a very good position to directly scavenge reactive oxygen species (ROS) formed by photochemistry (Agati et al. 2007). Transcription factors of flavonoid biosynthesis have been reported to be influenced by changes of the plant cell redox potential (Czemmel et al. 2009; Agati and Tattini 2010) and Pollastri and Tattini (2011) emphasize the potential of flavonols to contribute to the control of stress-induced changes in the cellular redox homoeostasis. Although the relevance of flavonoids in general as antioxidants *in planta* has been questioned, it is acknowledged for chloroplast flavonols (Hernández et al. 2009).

Flavonols are widespread in the plant kingdom but the amounts detected in fruits and vegetables vary greatly due to seasonal changes and varietal differences (Crozier et al. 2007). Flavones are not as widely distributed as flavonols and mainly found in parsley, celery and some herbs (Crozier et al. 2007). Red leaf lettuce contains glycosides of the flavonol quercetin: quercetin-3-*O*-glucoside, quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside, quercetin-3-*O*-glucuro-

nide, and the flavone luteolin: luteolin-7-*O*-glucuronide (Llorach et al. 2008). The aglycones (flavonoid molecules without sugar moieties) are depicted in fig. 3.

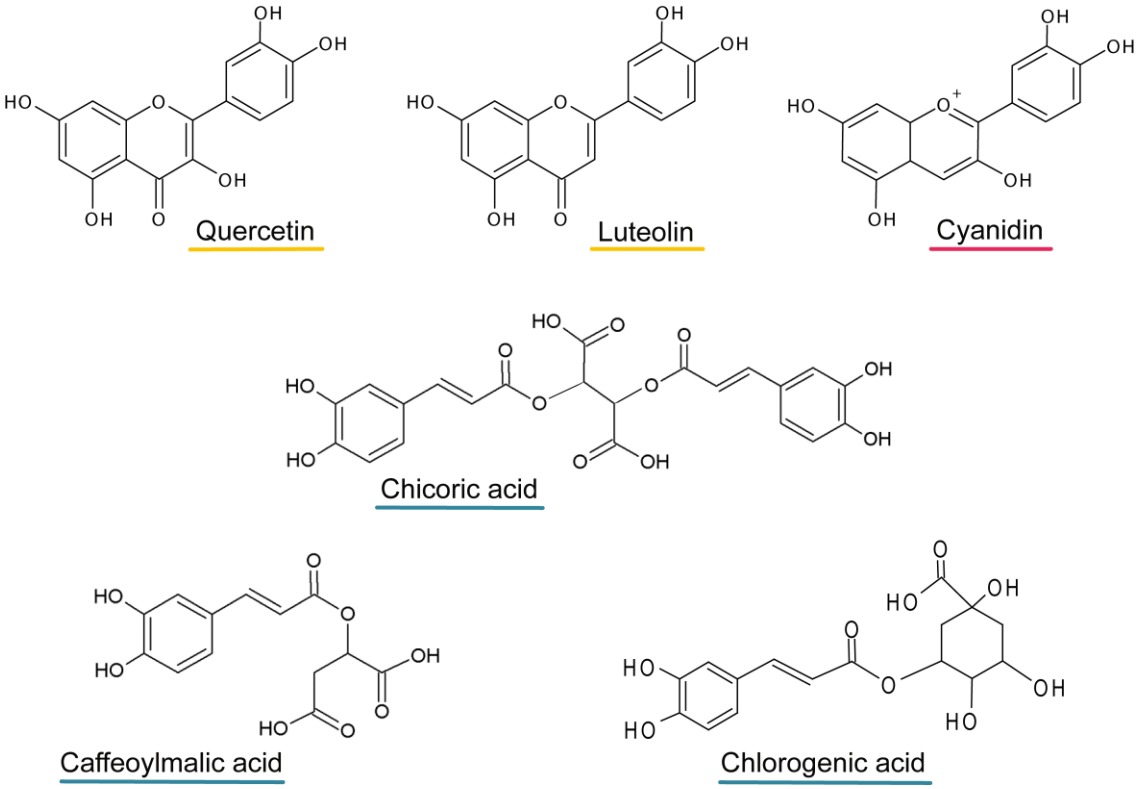

Figure 3: Major flavonoid aglycones and caffeic acid derivatives in red leaf lettuce: quercetin, luteolin, cyanidin, chicoric acid (di-*O*-caffeoyltartaric acid), chlorogenic acid (5-*O*-caffeoylquinic acid), *O*-caffeoylmalic acid. Compound names are supported by colored lines which are pointing out the different chemical classes. Throughout this work, flavonols and flavones will be highlighted in yellow, anthocyanins in red, and caffeic acid derivatives in blue.

Anthocyanins (= anthocyanidin glycosides) have been referred to as “nature’s swiss army knife” because of their versatility (Gould 2004). These cationic flavonoids (oxonium ion in C ring) provide a color palette from red to purple and blue dependent on pH or chelate formation with metal ions (Vogt 2010). Hereby they enhance flower and fruit attractiveness visually, thus facilitating pollination and seed dispersal (Gould 2004). Although non-toxic to mammalian and most insect grazers, anthocyanins can provide visual camouflage to deter herbivores (Gould and Lister 2006): To mammals which can perceive red light, the combination with green chlorophyll molecules results in a brown color (fig. 4) camouflaging leaves as being dead or even making them invisible against soil. Insects like aphids, however, lack red light receptors but are

attracted to green-yellow light which is effectively absorbed by anthocyanins, rendering leaves invisible/ unattractive.

Throughout the plant kingdom anthocyanins are widespread, providing high antioxidant and radical scavenging activity (Rice-Evans et al. 1997; Neill and Gould 2003). Foliar anthocyanins also have a photoprotective feature: By absorbing radiation from the green and yellow wavebands they protect photosynthetically active cells from excess energy that would otherwise lead to photoinhibition, i.e. decline of photosynthetic quantum yield in strong light, and photooxidation, i.e. light induced oxidative damage (Hatier and Gould 2009). Additionally they absorb UV radiation.

There is an ongoing debate whether or not there is a unified explanation for the occurrence of anthocyanins in leaves: Points are made for the photoprotective feature to be paramount while others focus on the antioxidant protection or their osmoregulatory role because water stress is induced by all kinds of suboptimal conditions (Gould and Lister 2006). Anthocyanins are found in varying amounts in plants. Among the list of anthocyanin rich vegetable there are red onion, eggplant, red cabbage, and black beans to name only some (Wu and Prior 2005b). Red leaf lettuce contains one anthocyanidin glycoside: cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside (Llorach et al. 2008). The cyanidin aglycone is depicted in fig. 3.

Figure 4: Leaf of red Lollo lettuce. Anthocyanins (dark color) have only accumulated in cells that were exposed to radiation. Although cyanidin-3-*O*-(6''-*O*-malonyl)-glucoside is a red pigment, the anthocyanic leaf areas appear brown in red lettuce because of the green chlorophyll molecules underneath.

Protection against UV radiation and counteracting reactive oxygen species both also appear to be functions of caffeic acid derivatives and other hydroxycinnamic acid related compounds (Edreva 2005b). Which of these roles predominates has been subject to discussion (Niggeweg et al. 2004; Tattini et al. 2004). Variation between plant species regarding their phenolic acid content and profile – which is additionally influenced by environmental factors – may to some degree account for controversial results (Clé et al. 2008).

Red leaf lettuce contains chlorogenic acid (5-*O*-caffeoylquinic acid), chicoric acid (di-*O*-caffeoyltartaric acid) and caffeoylmalic acid (Llorach et al. 2008) which are depicted in fig. 3. Chlorogenic acid is fairly well studied in plants. It clearly acts as antioxidant and is likely to protect against UV radiation, additionally to being involved in defense against pathogens (Niggeweg et al. 2004; Tegelberg et al. 2004; Clé et al. 2008). Chicoric acid and caffeoylmalic acid are not yet as well studied and there is no data specifically studying their roles in plants. Yet, if the caffeic acid moiety provides for UV absorption and antioxidant activity as suggested in the literature, these two compounds are likely to display similar traits to chlorogenic acid.

Although the importance of each group of phenolic compounds varies across plant species as well as between leaves of different developmental stages, flavonoid biosynthesis genes are present in plant species from all orders of the plant kingdom – from the basal liverworts to the most advanced angiosperms (Gould and Lister 2006).

Flavonoids have possibly been evolutionary established after the plant colonization of land over 400 million years ago. They may have enabled plants to live in an environment posing the threat of desiccation and being richer in oxygen and ultraviolet radiation than their previous aquatic habitat – all bearing the danger of oxidative damage. Their field of activity may have been extended only later on to perform an array of different tasks, accomplishing nature's tendency to "kill as many birds with one stone" as possible. This hypothesis has been put forward by several authors (Gould and Lister 2006; Pollastri and Tattini 2011; Agati et al. 2012).

1.3 Biosynthesis and structure of flavonoids and caffeic acid derivatives

According to Crozier et al. (2007) and Treutter (2010), biosynthesis of flavonoids and hydroxycinnamic acids in plants occurs in several steps involving the shikimic and the phenylpropanoid pathway, providing L-phenylalanine and *p*-coumaric acid, respectively, as important intermediates (fig. 5).

The C_6-C_3-C_6 flavonoid skeleton (fig. 2) is derived from phenylpropanoid pathway (bridge and aromatic B ring) as well as the malonate pathway (aromatic A ring). For synthesis of the bridge and aromatic B ring, D-glucose is in several steps transformed into shikimic acid, quinic acid, and then L-phenylalanine. By removal of the amino group cinnamic acid is formed and subsequently converted to *p*-coumaric acid and then to *p*-coumaroyl-CoA.

Via acetyl-CoA, three malonyl-CoA units are derived from the carbohydrate metabolism to form the A ring through a stepwise condensation with *p*-coumaroyl-CoA. The resulting chalcone is subsequently transformed into a flavanone which can then be turned into a flavone (e.g. luteolin) or be converted into a dihydroflavonol and then a flavonol (e.g. quercetin). Alternatively, the dihydroflavonol can be transformed into leucocyanidin, a precursor of anthocyanidins like cyanidin. The majority of flavonoids naturally occurs as glycosides (Crozier et al. 2007). Sugar moieties are introduced after the aglycones have been synthesized. In lettuce, quercetin and cyanidin occur as 3-*O*-glycosides and luteolin as 7-*O*-glycosides which are the most common forms of glycosylation for flavonols, anthocyanidins, and flavones.

The enzymes phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), and anthocyanidin synthase (ANS) are key enzymes (see fig. 5). While the first one catalyzes the conversion of L-phenylalanine into cinnamic acid, thus connecting the shikimic and the phenylpropanoid pathway, the second one catalyzes the condensation of *p*-coumaroyl-CoA with 3 malonyl-CoA, thus introducing flavonoid synthesis. The third one is key in cyanidin synthesis.

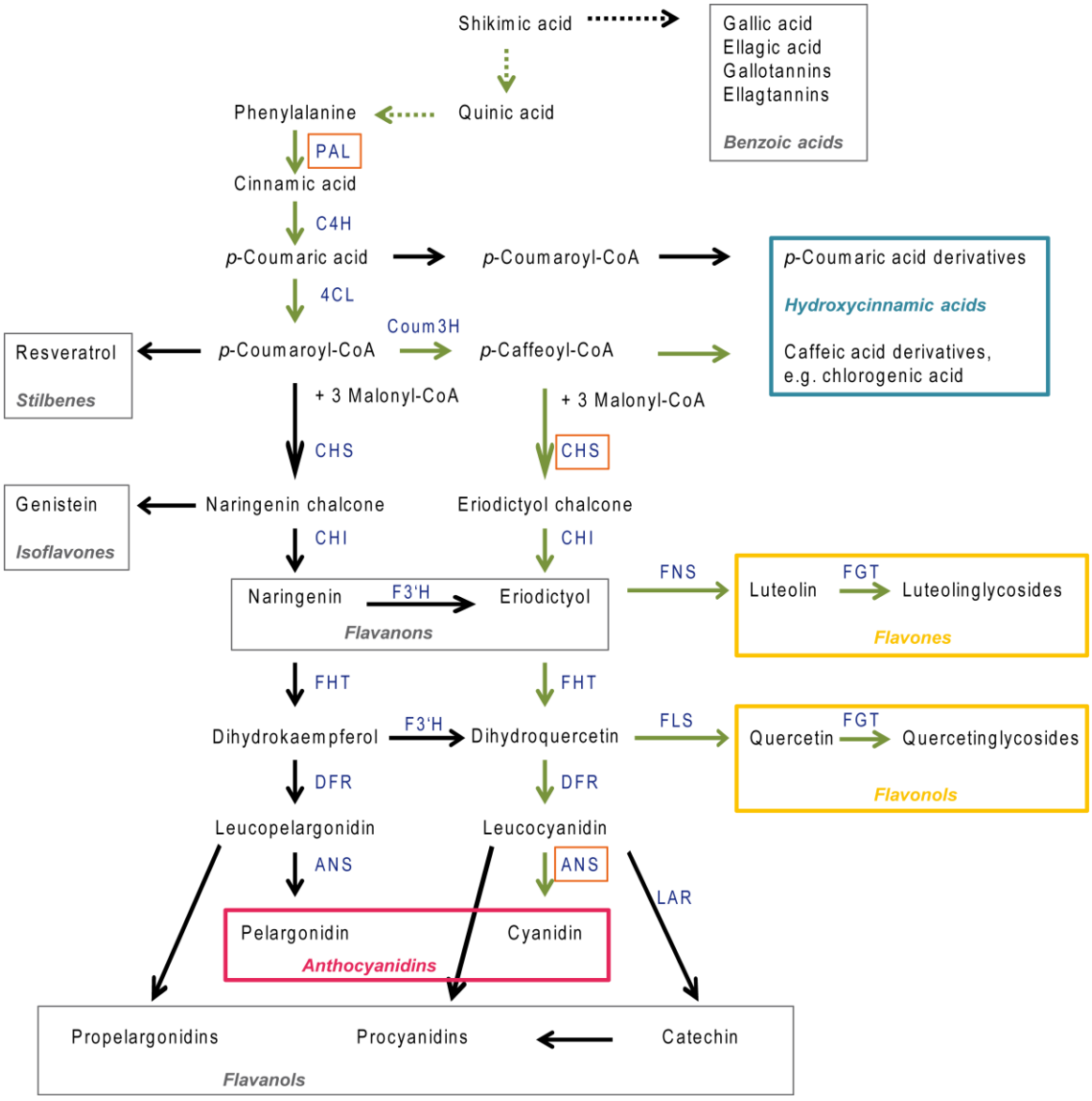

Figure 5: Biosynthesis of flavonoids and phenolic acids in a simplified scheme (Treutter (2010), modified). Green arrows mark the reactions relevant for compounds that can be found in lettuce. The respective compound groups are marked by colored boxes. Enzymes are indicated in dark blue letters. The key enzymes PAL (phenylalanine ammonia lyase), CHS (chalcone synthase), and ANS (anthocyanidin synthase) are highlighted by orange boxes. C4H: cinnamate 4-hydroxylase, CHI: chalcone isomerase, 4CL: *p*-coumarate:CoA ligase, Coum3H:coumaroyl 3-hydroxylase, DFR: dihydroflavonol 4-reductase, F 3'-H: flavonoid 3'-hydroxylase, FGT: flavonoid glycosyltransferase, FHT: flavanone 3-hydroxylase, FLS: flavonol synthase, FNS: flavone synthase, IFS: isoflavone synthase, LAR: leucocyanidin reductase

Caffeic acids are also synthesized via shikimic and phenylpropanoid pathway but take a different branch than flavonoids: *p*-coumaroyl-CoA can enter the flavonoid biosynthesis or alternatively be converted to caffeoyl-CoA and coupled with quinic acid to form 5-*O*-caffeoylquinic acid (Niggeweg et al. 2004). There are no studies on the biosynthesis of chicoric acid (di-*O*-caffeoyltartaric acid) and caffeoylmalic acid. As they are also caffeic acid derivatives, they may be synthesized similarly to chlorogenic acid but by coupling tartaric acid and another unit of caffeoyl-CoA and malate, respectively, instead of quinic acid. However, there has been a debate about the biosynthesis of caffeic acid derivatives highlighting differences between species (Niggeweg et al. 2004) and there is no certainty if the postulated biosynthetic mechanisms for caffeic acid derivatives apply to lettuce.

Anthocyanins, flavonols and caffeic acid derivative biosynthesis is regulated on a single cell basis depending on stimuli (Steyn et al. 2002; Edreva 2005b) as demonstrated by the foliar coloration due to anthocyanin distribution in fig. 4.

1.4 Nutritional quality of leaf lettuce – Is it healthy?

A diet rich in polyphenols has been associated with lower incidence of coronary heart disease and cancer by epidemiological studies (Boudet 2007). Especially flavonol and flavone intake had shown protective effects on coronary arteries and lungs, as summarized by Crozier et al. (2009). These effects have been ascribed to their ability to scavenge free radicals and protect the body's tissue from oxidative stress, thus preventing the onset of many diseases (Scalbert and Williamson 2000). Lately a more complex picture has emerged: Polyphenols have been observed to directly inhibit cancer cell proliferation, cholesterol uptake, act anti-inflammatory, interact with several signal transduction pathways and specifically with many enzymes (Buer et al. 2010).

Major phenolic compounds in red leaf lettuce are the aforementioned quercetin-3-*O*-glucoside, quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside, quercetin-3-*O*-glucuronide, luteolin-7-*O*-glucuronide, cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside, chicoric acid, chlorogenic acid, and caffeoylmalic acid (Llorach et al. 2008). I*n vitro* studies have found impressing effects for several of these compounds. In detail, quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside was observed to have antioxidative and antiatherogenic effects (Enkhmaa et al. 2005). The quercetin aglycone can attenuate memory damage in mice, presumably by inhibiting peroxidation of polyunsaturated fatty acids of the neuronal cell membrane lipids (Choi et al. 2012). Cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside and chicoric acid inhibited lipid peroxidation and cyclooxigenase enzymes (Mulabagal et al. 2010). Chicoric acid additionally showed HIV-integrase inhibitory activity (Lee et al. 2007). Chlorogenic acid has the ability to protect against induced carcinogenesis by interfering with involved signaling pathways, for instance by interacting with mitogen-activated protein kinase, nuclear factor kappa B, and activator protein-1 (Niggeweg et al. 2004; Crozier et al. 2009).

Apart from effects of single phenolic compounds, interactive effects and synergisms of dietary phenolic compounds have received increasing attention in order to elucidate the positive effects of a diet rich in polyphenols (Boudet 2007). In this context, a synergistic effect of chicoric acid and luteolin has been reported: Chicoric acid is a potent antioxidant while luteolin has anti-inflammatory effects by inhibiting several inflammatory mediators - When administered together, these

two substances show a stronger anti-inflammatory effect than luteolin alone (Park et al. 2011).

In order for these impressing effects to take action *in vivo* these compounds have to be bioavailable. This term is derived from pharmacology and describes the extent to which an ingested nutrient or compound reaches systemic circulation and specific sites to exert its biological action (D'Archivio et al. 2010). Discussion about the bioavailability of polyphenols in humans has been long ongoing (Scalbert and Williamson 2000; D'Archivio et al. 2010) and has very recently been reviewed by (Del Rio et al. 2013).

Chlorogenic acid (5'-*O*-caffeoylquinic acid) is highly bioavailable for the human metabolism (Niggeweg et al. 2004). The most probable mechanism is the absorption of caffeic acid released from chlorogenic acid after cleavage by esterases of the gut microflora (Plumb et al. 1999; Nardini et al. 2002; Manach et al. 2005). Its antioxidant capacity is comparable to that of chlorogenic acid (Rice-Evans et al. 1997). A very similar mechanism may apply to chicoric acid: It comprises tartaric acid esterified to two caffeoyl moieties which could be released by gut esterases.

Mostly, quercetin and cyanidin are attributed a rather poor bioavailability due to their bad membrane-permeability (Manach et al. 2005). Yet, cyanidin-3-*O*-glucoside as well as cyanidin-3-*O*-(6''-*O*-malonyl)-glucoside were found to be absorbed in the stomach and intestine of rats and their derivatives were recovered in the urine (Felgines et al. 2006) and data collected by Scalbert and Williamson (2000) further displays the heterogeneity of results on the bioavailability of quercetin and its glucosides: Maximum concentration in the plasma ranged from 0.15 to 2.22% of the ingested quantity, possibly owing to their structure (as aglycone or as glycoside, different sugars constituting the glycosidic moiety). Furthermore, if they were not ingested as pure compound interactions with the food matrix, i.e. other dietary components like carbohydrates, proteins, and fats, can influence bioavailability (D'Archivio et al. 2010). Bioavailability studies are often conducted administering single doses, not including the more realistic scenario of regular intake of low amounts of polyphenols which could lead to an increased concentration on plasma and cell level (D'Archivio et al. 2010).

Possible ingestion mechanisms for flavonoid glycosides are reviewed by Galvano et al. (2004) and D'Archivio et al. (2010), suggesting transport into small intestine epithelian cells by sodium-dependent glucose transporter 1 or glycoside hydrolyzation by the β-glucosidase lactase phloridizin hydrolase present in the small intestine and colon of humans followed by passive diffusion.

Yet, regardless of bioavailability, dietary polyphenols may be beneficial for human health by preventing peroxidation of dietary lipids in the stomach during ingestion (Gorelik et al. 2005).

Still, caution is needed when increasing polyphenol intake: At high concentration, antioxidants of the flavonol-type can act as pro-oxidants: Their quinone-type metabolites can exhibit mutagenic character by forming DNA adducts (Rietjens et al. 2005). While overdosing by vegetable consumption is unlikely, flavonoid containing food supplements are often viewed critically (Skibola and Smith 2000). Considering danger of overdosing on the one hand and the possible synergistic effects of dietary polyphenols on the other hand, it appears wise to generally enhance the accumulation in fruits and vegetables by horticultural approaches instead of administering single substances as dietary supplement. In order to do this we need to understand how their biosynthesis responds to different cultivational strategies. So far, experiments investigating polyphenol biosynthesis and how it is affected by ecophysiological factors often focused on extreme situations rather than realistic cultivational approaches.

The apprehension that increased polyphenol concentrations in lettuce could decrease the sensory quality by increasing its slightly bitter taste is unsubstantiated (Bunning et al. 2010). The specific bitter taste found in some cultivars is not attributed to polyphenols but to lactucin, a sesquiterpene lactone, and hyoscyamin, a tropane alkaloid (Krug et al. 2002).

Well, can lettuce be considered healthy food? Baslam et al. (2013) support this notion, based on the contained minerals and potential antioxidants. It may not be the vegetable accumulating highest concentration of polyphenols: Onion for instance way outranges lettuce (Chu et al. 2000). Nevertheless, it can serve as a good dietary source because it is commonly consumed raw and in large quantities (Clifford 2000). Especially in its outer leaves, leaf lettuce contains

higher concentrations of health promoting compounds than head lettuce (Mou 2009; Baslam et al. 2013).

1.5 ZINEG-project: Measures to reduce greenhouse energy consumption

In Central Europe, lettuce is cultivated in greenhouses in cool seasons. Unfortunately, conventional greenhouses consume a lot of energy: Besides labor, energy consumption is accountable for a substantial share of costs during crop production (Meyer 2011). With increasing prices for fossil fuel, enhanced public interest in CO_2 balances of food and political will to reduce CO_2 emissions, producers can face enormous pressure and a need for new approaches and ideas arises (Bakker et al. 2007; Frangi et al. 2011; Meyer 2011).

The German ZINEG project (www.zineg.de) is developing strategies to operate greenhouses more climate friendly, idealistically CO_2 neutral, using a system-oriented approach which combines technical innovations and cultivational measures (Tantau et al. 2009). Application of transparent energy saving screens, for example, features a large energy saving potential – unfortunately, they also reduce the photosynthetically active radiation (PAR) available for crops (Bakker et al. 2007). Hence, photosynthesis is reduced and less biomass accumulation results in lower yields (Klaering and Krumbein 2013).

Another obvious approach to reduce energy consumption of greenhouses is to lower the cultivation temperature. This influences plants in manifold ways: Decreasing temperature generally slows down metabolic processes. With lettuce, this results for example in delayed growth, hence postponed development of marketable lettuce heads (Wurr et al. 1996).

Yet, plant growth is only one of many aspects affected by low temperature-cultivation or reduced irradiation due to energy saving screens. Improving greenhouse crop production from ecologic and economic viewpoints is of great importance. Nevertheless, we must not lose sight of vegetables being our basic diet and, thus, closely related to our health (Martin et al. 2011). Phytonutrients are influenced by growing conditions and low temperature as well as low radiation cultivation are likely to have an impact on the polyphenol concentration of red leaf lettuce (Treutter 2010).

1.6 How is the concentration of flavonoids and phenolic acids influenced by radiation?

Light intensity directly influences the light-dependent part of photosynthesis, the overall rate of photosynthetic processes, and therefore the rate of ROS formation (Edreva 2005a). Flavonoids have been linked to the redox homoeostasis of plant cells (see section 1.2). Hence, an effect on flavonoid concentration is very likely. Concordantly, the expression of phenylalanine ammonia lyase is light dependent (Leyva et al. 1995).

UV radiation and high photosynthetic photon flux density (PPFD) increase the concentration of flavonoids in lettuce (García-Macías et al. 2007; Oh et al. 2009; Tsormpatsidis et al. 2010). Furthermore, lettuce plants contain higher concentrations of flavonols when grown in the field than when cultivated in a greenhouse (Romani et al. 2002) which may to some extend be explained by differing radiation intensities and spectra as greenhouse glass absorbs some PAR and large percentages of the incident UV radiation (see appendix, p. 122-124, fig. 49 and tab. 6 for details).

Liu et al. (2007) measured higher total phenolic content and radical scavenging ability in leaf lettuce extracts than in extracts of head forming lettuce types. The cause may be their morphology: In leafy lettuce a larger surface area is exposed to light. Hohl et al. (2001) demonstrated that the comparably low flavonoid concentration in inner lettuce leaves can be increased by exposing them to radiation. Yet, there is no study on the effect of shading (i.e. low PPFD) on flavonoid glycosides in lettuce heads. A study carried out with tea (*Camellia sinensis* L.) leaves found a significant decrease of *O*-glycosylated flavonol concentration due to shading in comparison to full sunlight conditions, supported by gene expression analysis of the respective enzymes of the phenylpropanoid pathway (Wang et al. 2012).

In in *Ligustrum vulgare* L. leaves, *p*-coumaric acid and caffeic acid derivatives were not influenced by the reduction of solar radiation (Agati and Tattini 2010). Yet, in response to UV radiation and short term high PAR, caffeic acid derivatives were demonstrated to accumulate in lettuce (García-Macías et al. 2007; Oh et al. 2009).

1.7 How is the concentration of flavonoids and phenolic acids influenced by plant ontogeny?

During ontogeny, plants live through different phases with distinct morphological and physiological features (Hackett 2002). Although crop plants are often harvested before they have transitioned into the generative phase or completed reproduction, their development can be divided into growth stages with pronounced differences regarding morphology and physiology. The concentration of flavonoids and phenolic acids is known to be subject to variation throughout plant development in many crops (Krumbein et al. 2007; Treutter 2010).

Because it is commonly consumed as leafy vegetable, lettuce is in fact harvested before it enters generative growth. Nonetheless, there are distinguishable growth stages related to head formation. This process is based on leaf re-orientation from horizontal towards an upright position, progressively bending inwards to form a head that will grow and become larger and denser with an increasing number of leaves (see section 1.1). This affects for instance the distribution of radiation within the plant: Unlike those on the outside, leaves inside of the head will not be directly exposed to radiation or only at their tips. Due to the light-dependency of flavonoid biosynthesis, their concentration is higher in outer than in inner leaves (Hohl et al. 2001).

In red leaf lettuce, young leaves have lower quercetin content than older ones (Behn et al. 2011) and data published by Romani et al. (2002) suggests that the overall concentration of flavonol glycosides and caffeic acid derivatives of whole heads is higher in lettuce plants in early compared to later growth stages. However, they focused on comparing greenhouse and open field grown lettuce and did not report detailed information on growing conditions while anthocyanins were not included in their analyses at all.

When it comes to studying development-dependent effects of radiation on plants, many studies have focused on UV radiation (Kubasek et al. 1998; Reifenrath and Müller 2007; Behn et al. 2011). The light-dependency of flavonoid biosynthesis is well known. Additionally, Kubasek et al. (1998) found the developmental stage to be influencing the induction of pivotal flavonoid biosynthesis genes in *Arabidopsis thaliana* seedlings. Furthermore, Reifenrath

and Müller (2007) detected an interaction between UV radiation and leaf age regarding their effect on flavonol concentration in *Nasturtium officinale* (Brassicaceae). This indicates that the same elicitor could lead to a different response from the same plant – depending on the plant's growth stage. Indeed, flavonoids in red leaf lettuce display a development-dependent response to UV radiation (Behn et al. 2011).

1.8 How is the concentration of flavonoids and phenolic acids influenced by low temperature?

Low temperature slows down every metabolic process. In plants, this general deceleration affects for example the Calvin cycle enzymes of the light-independent part of photosynthesis (Havaux and Kloppstech 2001). As a result, the supply of nicotinamide adenine dinucleotide phosphate ($NADP^+$), the final electron acceptor of the photosynthetic electron transport chain (ETC), can be limited (Pfannschmidt 2003). Yet, as light energy interception is not decelerated and the electron carriers may eventually be overreduced, electrons can leak to molecular oxygen instead of $NADP^+$, i.e. produce reactive oxygen species (Havaux and Kloppstech 2001; Pfannschmidt 2003). ROS have the potential to destroy thylakoid membranes (the site of the light-dependent photosynthetic reactions), damage DNA, and denature proteins (Gould et al. 2002). Detrimental effects of low temperature-induced oxidative damage may be enforced by enzymatic repair processes being also slowed down (Bilger et al. 2007). However, ROS themselves can be perceived by plants and act as messenger molecules, eventually influencing gene expression and conveying acclimation to an altered environment (Edreva 2005a; Gill and Tuteja 2010).

Flavonoid biosynthesis increases with lower temperatures in European winter crops (Neugart et al. 2012a). This was also found in growth chamber experiments with cool-cultivated *Arabidopsis thaliana* (Havaux and Kloppstech 2001). However, there are only few studies on the effect of temperature on the phenolic compounds in lettuce (Gazula et al. 2005; Oh et al. 2009; Boo et al. 2011). None of them addressed the long term effect of low temperature on all major phenolic compounds in red leaf lettuce: Oh et al. (2009) only applied low temperature for one day. Gazula et al. (2005) subjected plants to temperature treatments for 20 days but investigated only the accumulation of anthocyanins

and in a higher temperature range (20 – 30 °C). Boo et al. (2011) cultivated plants for six weeks but only measured anthocyanins and total polyphenols. Furthermore, they did not take into account that together with varying temperature, the plants' growth rates vary (Wurr et al. 1996). This may be critical because data published by Romani et al. (2002) suggest higher concentrations of quercetin glycosides and caffeic acid derivatives in lettuce in early growth stages compared to later ones. The relevance of head development for the concentration of quercetin glycosides has been reported for other vegetables (Krumbein et al. 2007) and may also apply to anthocyanins.

Data on the response of phenolic acid biosynthesis to low temperatures is less consistent. Some studies report increasing phenolic acid concentration with low temperatures (Zidorn 2010), some find no effect of temperature alone but rather in combination with other abiotic factors (Grace et al. 1998; Løvdal et al. 2010) while others find different phenolic acids to respond disparately (Oh et al. 2009).

2 Objectives

Conventional greenhouses in Central Europe consume large amounts of energy (Meyer 2011). The application of transparent energy saving screens features a large saving potential but unfortunately reduces the PAR available for crops (Bakker et al. 2007). Another approach to reduce greenhouse energy consumption is to cultivate at lower temperature. Both reduced PAR and low cultivation temperature influence plant growth as well as secondary metabolites (Treutter 2010).

Objective of this work was to determine to which degree these altered conditions would influence flavonoid glycosides and caffeic acid derivatives in red leaf lettuce (*Lactuca sativa* var. *crispa*). Lettuce is an important food crop in the European Union and usually cultivated in greenhouses in cool seasons when the mentioned energy saving approaches would be applied (Krug et al. 2002; Baslam et al. 2013). Red leaf lettuce displays an interesting polyphenolic profile of cyanidin, quercetin, and luteolin glycosides as well as caffeic acid derivatives (Llorach et al. 2008).

Reduced radiation leads to decreased concentrations of *O*-glycosylated flavonols in *Ligustrum vulgare* and *Camellia sinensis* (Agati and Tattini 2010; Wang et al. 2012). Yet, the effect of shading on flavonoid glycosides and caffeic acid derivatives in lettuce has not been studied. Existing studies only investigated the short term effect of PPFD as high as 800 µmol m^{-2} s^{-1}on young lettuce plants (Oh et al. 2009) or the effect of UV radiation (Tsormpatsidis et al. 2010). Phenolic acids were reported to respond to short-term high PAR or UV radiation (García-Macías et al. 2007; Oh et al. 2009) while other authors found no effect (Agati and Tattini 2010). Yet, none of these approaches is relevant for the above described scenario. The effects of long and short term treatments may differ substantially. Furthermore, the impact of low level may be very different from high level PPFD. The heterogeneity of data on the response of phenolic acids to radiation additionally accentuates the need for further attentive research in this area.

Alternatively to screen application during the whole cultivation period, it would be energetically worthwhile to apply screens only part of the time. Lettuce has the capacity to make up for shading-induced growth shortfalls if subsequently

there is a sufficiently long unshaded phase (Sanchez et al. 1989). It is unclear if polyphenols in lettuce respond accordingly.

Experiment 1 addressed the question if reduction of an already non-stressful PPFD level (410 to 225 µmol m^{-2} s^{-1}) at standard cultivation temperature (20/ 15 °C, day/ night) results in decreased concentrations of flavonoid glycosides and caffeic acid derivatives in red leaf lettuce. In growth chambers, the influence of reduced PPFD was tested in an early and a more advanced growth stage additionally to cultivating plants continuously shaded or unshaded in order to detect less sensitive growth stages. Applying temporary as well as continuous shading to compare the long-term dynamics of the phenolic status of lettuce is a new approach that has not been used before.

Lettuce lives through physiologically and morphologically distinct growth stages (Krug et al. 2002). Although in many crops flavonoid and phenolic acid concentration changes during ontogeny (Krumbein et al. 2007; Treutter 2010), it has never been studied if the response to abiotic factors is likewise related to growth stage in lettuce. In its vegetative phase, the process of head formation constitutes major changes in plant architecture, affecting at least the distribution of radiation within the plant. Additionally, varying induction potential of genes whose products are involved in flavonoid biosynthesis in *Arabidopsis thaliana* (Kubasek et al. 1998) indicates that – depending on growth stage – the same elicitor could induce different responses from the same plant. Indeed, flavonoids in young and old lettuce leaves respond differently to short term exposure to UV radiation (Behn et al. 2011). Yet, it is unclear if this interaction between radiation and leaf age also shows on the whole head-level, when plants are cultivated under low levels of PPFD, and if it also applies to caffeic acid derivatives.

Experiment 2 investigates if flavonoid glycoside and caffeic acid derivative concentrations respond to PPFD levels (230 - 43 µmol m^{-2} s^{-1}) as low as would be expected in greenhouses in cool seasons in Central Europe and if the plants' growth stage is relevant for their response to radiation. The experiment was conducted in the greenhouse, plants were harvested in three different growth stages. In a novel approach, data obtained on their respective concentration of flavonoid glycosides and caffeic acid derivatives was evaluated via multiple regression analysis.

Temperature has been found to influence the concentration of flavonoid glycosides and caffeic acid derivatives in lettuce (Gazula et al. 2005; Oh et al. 2009; Boo et al. 2011). However, none of these studies investigated the long term effect of low temperature on these compounds. Furthermore, none of them have taken into account that plant growth rates vary depending on temperature (Wurr et al. 1996). Yet, this may be crucial in long term studies as comparing plants in different growth stages can introduce a large bias.

Regarding the response of phenolic acids to low temperatures, some studies report increasing phenolic acid concentrations while others detected no influence or only in interaction with other factors (Grace et al. 1998; Oh et al. 2009; Løvdal et al. 2010; Zidorn 2010). Clearly, more and attentive research is needed here.

Experiment 3 was conducted in growth chambers with a day/ night temperature of 20/ 15 °C and 12/ 7 °C, respectively. The mean PPFD was 247 µmol m^{-2} s^{-1} during the day. Thus, the warm treatment is comparable to the shade treatment in experiment 1. The concentration of flavonoid glycosides and caffeic acids in plants cultivated cool or warm were compared. In order to detect growth stages that are less temperature sensitive and to test the dynamics of possible low temperature induced changes in the plants' phenolic status, plants were exposed to low temperature temporary in an early and in a more advanced growth stage, additionally to exposing plants continuously to either the cool or the warm temperature regime. The effect of temperature can vary during ontogeny (Wheeler et al. 1993) and already part-time low temperature cultivation would be energetically worthwhile.

A new approach was implemented to avoid the developmental bias when studying the long term influence of low temperature on flavonoid glycosides and caffeic acids in red leaf lettuce: Harvest dates were determined based on the concept of accumulated thermal time instead of elapsed time (Tei et al. 1996). The harvest schedule so composed allowed for obtaining information on plants in comparable growth stages which they reached after a different number of days in differing temperature regimes (Tei et al. 1996; Wurr et al. 1996).

All three experiments were conducted until plants reached marketable head weight in order to gain results of practical relevance. Additionally, plant growth stages were taken into account and compounds were analyzed carefully and detailed via HPLC-DAD-ESI-MS^n. Experiment 1 and 3 were conducted in growth chambers to strictly separate the effects of temperature from radiation because they are known to interact (Løvdal et al. 2010). Experiment 2 was conducted in the greenhouse to approach realistic lettuce production conditions.

In contrast to previous studies, all investigated PPFD levels were non stressful (Fu et al. 2012) but covering a wide range (43 - 410 µmol m^{-2} s^{-1}). This is more interesting to horticulture than just studying extreme short term situations

This thesis offers profound analytical chemistry investigating horticulturally meaningful scenarios, discussed in a plant physiological context.

3 Material and Methods

3.1 Plant cultivation

3.1.1 Influence of radiation – Experiment 1: Growth chamber

Red Oak Leaf lettuce (*Lactuca sativa* L. var. *crispa* L. cv. Eventai RZ, RijkZwaan, De Lier, The Netherlands) was sown in rockwool cubes (4 cm x 4 cm x 4 cm), kept at ca. 10 °C for two days for germination and subsequently grown in a conventional greenhouse until the experiment started. When plants had developed six to seven true leaves and had a mean aboveground mass of 1.4 g (5 weeks after sowing) they were transferred into growth chambers (Yorck, Mannheim, Germany) where they were grown hydroponically using deep flow technique in three growth chambers simultaneously.

Nutrient solution was prepared according to Sonneveld and Straver (1988) and exchanged and checked for macro nutrients every week. Air temperature was 20 °C during daytime and 15 °C at night. Relative humidity was 85 - 90% during daytime and 80% at night. Radiation was supplied by high-pressure sodium discharge lamps SON-T PLUS 400 W (Philips, Amsterdam, The Netherlands; see appendix, p. 123, fig. 48 for spectrum).

In each growth chamber half of the plants were grown under a net (mesh size: 0.25 cm²) which reduced the PPFD on average by 45% (see fig. 6 for experimental setting and appendix, p. 122, tab. 6, for details on transmittance of the net). Mean PPFD was 410 µmol m^{-2} s^{-1} for the uncovered plants and 225 µmol m^{-2} s^{-1} under the net, as measured with a portable light meter LI-180 (LI-COR Inc., Lincoln, Nebraska, USA). The light cycle consisted of four elements: 11 h of darkness, 0.5 h of dawn, 12 h of light and another 0.5 h of dusk. During “dusk” and “dawn”, respectively, only some of the lamps were switched on, resulting in a mean PPFD of 95 µmol m^{-2} s^{-1}. This adds up to a daily light integral of approximately 18 and 10 mol PAR m^{-2} d^{-1}, respectively, for the unshaded and the shaded treatment.

When the plants had been growing inside the chambers for 14 days, one third of them were exchanged between treatments, one third was harvested and one third stayed in their respective treatments. After 28 days all remaining plants were harvested. Thus, after 28 days there were four treatments which received

the following total light integrals: shaded: 280, unshaded: 504, first shaded then unshaded: 392 and first unshaded then shaded likewise 392 mol PAR m^{-2}.

Figure 6: Setting of experiment 1 in a growth chamber. Reduced PPFD was studied with oak leaf lettuce cultivated by deep flow technique with constant supply of nutrient solution. On the right hand side of this photograph, plants are shaded with a net which reduces the incident PPFD by 45%.

3.1.2 Influence of radiation – Experiment 2: Greenhouse

Red Oak Leaf and red Lollo lettuce (*Lactuca sativa* L. var. *crispa* L. cv. Eventai RZ and *L. sativa* L. var. *crispa* L., cv. Satine, respectively; RijkZwaan, De Lier, The Netherlands) are both suitable for greenhouse cultivation in cool seasons. The experiment was conducted in Grossbeeren (52°20'N, 13°18'E), Germany, from March – April, 2012. Mean temperature in the greenhouse was 16.3 °C (min: 11.7 °C, max: 23.6 °C). Lettuce was sown in rockwool cubes, kept at ca. 10 °C for two days for germination and subsequently grown in a conventional greenhouse until the experiment started. When plants had developed four true leaves and a mean aboveground mass of 0.9 g (5 weeks after sowing) they were transferred into the experimental setting where they were grown hydroponically using nutrient film technique.

Nutrient solution was prepared according to Sonneveld and Straver (1988) and checked for macro nutrient concentration every week. The greenhouse area was divided into four blocks according to differences in radiation intensity due to greenhouse construction elements. Additionally, half of each block was covered with a net reducing the photosynthetic photon flux by 45% (see fig. 7 for experimental setting and appendix, p. 122, tab. 6 for net transmittance details). This way data on flavonoid glycoside and caffeic acid derivative concentration in two cultivars of red leaf lettuce related to eight PPFD levels (ranging from 43 - 230 µmol m^{-2} s^{-1}) per harvest date was obtained.

The natural light cycle was followed without supplying additional lighting. PPFD was monitored permanently in each block with light meters LI 190SA Quantum (LI-COR Inc., Lincoln, Nebraska, USA) and recorded by a data logger type DT50 (DATATAKER, Victoria, Australia). Aboveground organs were harvested at three dates, providing data on three disparate growth stages within the horticulturally interesting vegetative growth phase: Harvest one took place 12 days after planting (DAP), before head formation started. Harvest two took place shortly after head formation started (21 DAP) and at harvest three mature heads had been formed (35 DAP). Harvest dates were set based on experience gained in previous experiments. Intercepted total PAR integrals at the three harvest dates ranged from 25 – 77 mol m^{-2} at 12 DAP, 57 – 192 mol m^{-2} at 21 DAP and

94 – 301 mol m^{-2} at 35 DAP. The large range is due to the eight different PPFD levels included.

Light use efficiency was calculated for plants before and after the onset of head formation. This value displays how much dry matter plants gained per intercepted mol of PAR. For each block and cultivar the intercepted PAR was calculated separately, based on the mean measured head diameter. The PAR absorbing surface area (m^2) was approximated by a circle. This area was multiplied with the PAR integral (mol m^{-2}) for the intervals between the first and second and the second and third harvest date, respectively. Light use efficiency (g mol^{-1}) was obtained as the ratio of the plants' dry matter gain (g) between the respective harvest dates and the corresponding intercepted PAR.

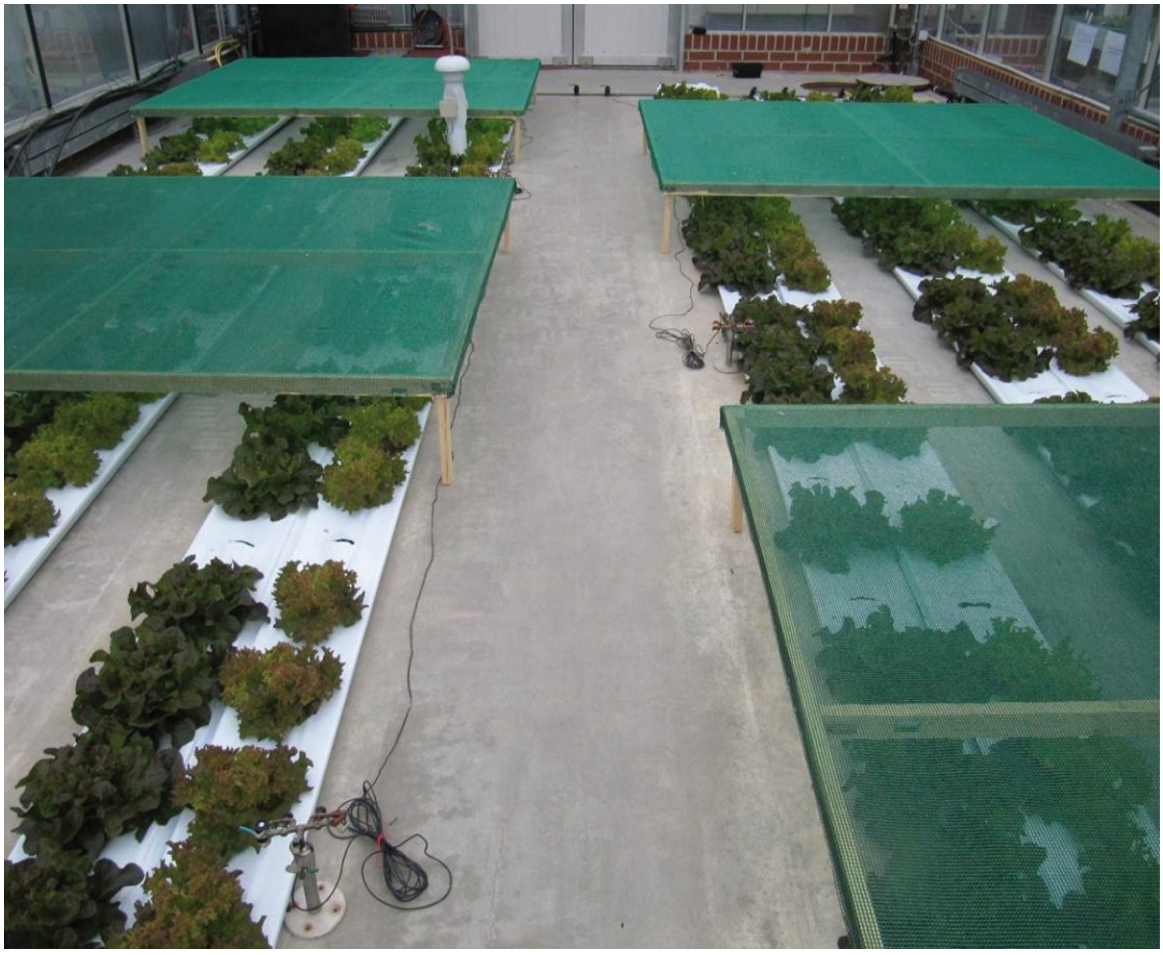

Figure 7: Experimental setting of experiment 2 in the greenhouse. Red Lollo and red Oak Leaf lettuce were cultivated at low level PPFD. The displayed nets reduce radiation by 45%. Additionally, there was a gradient of radiation due to greenhouse construction. PPFD was monitored permanently by light meters in the unshaded part of each block. Lettuce was cultivated by nutrient film technique.

3.1.3 Influence of temperature – Experiment 3: Growth chamber

Seeds of red Oak Leaf and red Lollo lettuce (*Lactuca sativa* L. var. *crispa* L. cv. Eventai RZ and *L. sativa* L. var. *crispa* L., cv. Satine, respectively; RijkZwaan, De Lier, The Netherlands) were sown in rockwool cubes (4 cm x 4 cm x 4 cm), kept at ca. 10 °C for two days for germination and subsequently grown in a conventional greenhouse until the experiment started. When plants had developed four true leaves (5 weeks after sowing) and weighed about 0.9 g they were transferred into growth chambers (Yorck, Mannheim, Germany) where they were grown in four growth chambers simultaneously using deep flow technique (see fig. 8 for experimental setting).

Nutrient solution was prepared according to Sonneveld and Straver (1988) and exchanged and checked for macro nutrients every week. In two chambers, air temperature was 20 °C during daytime and 15 °C at night (warm treatment), whereas it was 12/ 7 °C (day/ night) in the other two (cool treatment). Relative humidity was approximately 80%. Radiation was supplied by high-pressure sodium discharge lamps SON-T PLUS 400 W (Philips, Amsterdam, The Netherlands; see appendix, p. 123, fig. 48 for spectrum). The light cycle consisted of four elements: 11 h of darkness, 0.5 h of dawn, 12 h of light and another 0.5 h of dusk. During the light phase, mean PPFD was 247 µmol m^{-2} s^{-1}. During “dusk" and “dawn”, respectively, only some of the lamps were switched on, resulting in a mean PPFD of 95 µmol m^{-2} s^{-1}, as measured with a portable light meter LI-250 (LI-COR Inc., Lincoln, Nebraska, USA). Hence, plants intercepted a daily light integral of 11 mol PAR m^{-2} d^{-1}. Plants cultivated for 13 days intercepted a total light integral of 143 mol PAR, while those cultivated for 26, 39 and 52 days intercepted 286, 430, and 573 mol PAR m^{-2} s^{-1}, respectively.

In order to elucidate harvest dates at which the plants cultivated in different temperatures will have reached comparable growth stages (based on head mass and number of leaves) the concept of “sum of temperatures” was used. As rates of metabolic processes are temperature dependent, this concept uses accumulated thermal time instead of elapsed time to predict plant growth and development (Tei et al. 1996). Accumulated thermal time is measured in day-degrees (DD) and calculated by adding the values for daily mean temperature. This concept is widely used in horticultural crop production to predict harvest

dates and decide when to sow and plant. Based on previous experiments (data not shown), a target value of 400 DD was set (starting on the day of transfer into growth chambers) to obtain marketable lettuce heads of 200 - 250 g at the end of this experiment. Most crops have a “base temperature” below which no growth occurs. Based on previous experiments, a base temperature of 2 °C was assumed which was subtracted from the daily mean temperature in the calculations.

Figure 8: Setting of experiment 3 in a growth chamber. The effect of low compared to high temperature cultivation of red Lollo and red Oak Leaf lettuce was studied in four growth chambers simultaneously. Lettuce was cultivated by deep flow technique with constant supply of nutrient solution. Large plants on the left hand side of this photograph were already cultivated 13 days in a warm climate chamber and transferred into the cool chamber depicted here. Plants in the neighboring gully have been cultivated for the same number of days but in the cool chamber all the time.

The harvest schedule so composed allowed for obtaining information on plants in comparable growth stages which they reached after a different number of days in differing temperature regimes (Tei et al. 1996; Wurr et al. 1996). On

the other hand, lettuce plants cultivated at different temperature after the same number of days were harvested, in order to compare results to previous studies. The warm treatment reached the set day-degrees 26 days after planting (406 DD), the cool treatment 52 DAP (395 DD). Some plants were exchanged after they reached half of the day-degrees (203 and 198 DD, after 13 and 26 days in the warm and cool treatment, respectively). Plants in the exchange-variants were harvested 39 DAP (400 DD). On day 13 and 26 after planting, some plants were harvested from the warm and the cool treatment, respectively. Thus, at the end there was information about lettuce plants from the following six conditions and stages: small heads grown warm or cool (ca. 200 DD), as well as mature heads grown warm, cool, first cool then warm and first warm then cool (ca. 400 DD; see harvest schedule, fig. 9).

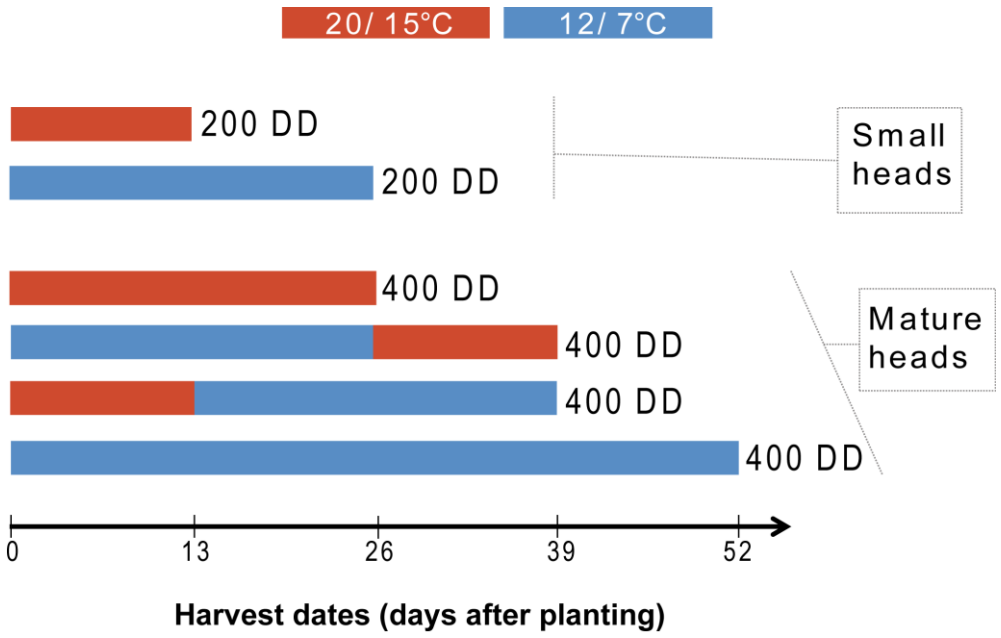

Figure 9: Harvest schedule based on accumulated thermal time, measured in day-degrees (DD). Red bars represent warm cultivation at 20/ 15 °C (day/ night), blue bars represent cool cultivation at 12/ 7 °C. Plants in the warm regime reached the DD set for harvest earlier than the plants in the cool regime. The target value for harvesting mature, marketable lettuce heads of 200 - 250 g was 400 DD. Some plants were exchanged between the warm and the cool growth chambers after they reached half of the DD aimed for (200 DD; 13 and 26 days after planting with warm- and cool-cultivated plants, respectively), in order to study the influence of temperature on lettuce in different growth stages. Two and four variants, respectively, were obtained: small heads cultivated either warm or cool as well as mature heads cultivated cool, warm, first cool then warm and vice versa. Thus it was possible to, on the one hand, compare them in corresponding growth stages and on the other hand compare cool- and warm-cultivated plants after the same number of days (26).

3.2 Head mass

In all three experiments, at all harvest dates, plants from each treatment, cultivar and replicate were weighed to obtain head mass. Values are given in gram fresh matter (FM).

3.3 Sample preparation

In all three experiments, a mixed sample from five plants was prepared for each treatment, cultivar, and replicate. Only limp or deteriorated outer leaves were removed. Within 30 minutes after harvesting the plants were cut in smaller pieces, mixed, and frozen at -20°C. About 300 - 400 g of fresh matter were frozen, lyophilized (Christ Beta 1-16, Osterode, Germany), and ground with an ultracentrifuge mill (hole size: 0.25 mm; ZM 200, Retsch, Haan, Germany). Weight before and after lyophilization was compared to obtain information on dry matter (DM) content.

3.4 Analyses of phenolic compounds

After optimization and validation (see 4.1), the following methods were used. For analysis of flavonol and flavone glycosides as well as caffeic acid derivatives, 0.5 g of lyophilized, ground lettuce were extracted with 25 ml of aqueous methanol (50% MeOH) at room temperature. The suspension was kept in motion with a magnetic stirrer for 1.5 h and then centrifuged for 15 minutes at 4500 rcf (relative centrifugal force; Labofuge 400R, Heraeus Instruments, Thermo Fisher Scientific, Waltham, USA). The supernatant was filtered with PTFE-syringe filters (0.25 µm, polytetrafluoroethylene; Roth, Karlsruhe, Germany), transferred into glass vials, and analyzed via HPLC-DAD-ESI-MS^n.

The anthocyanin extracts were prepared similarly, except for a slightly different composition of the extraction agent and shorter extraction time: The extraction agent was acidified aquaeous methanol (40% MeOH, 10% acetic acid), with a pH of 2.6. Extraction of anthocyanin glycosides took 15 minutes.

The system used for analysis consisted of an Agilent HPLC series 1100 (Agilent, Waldbronn, Germany), containing a degaser, binary pump, autosampler, thermostat, and a photodiode array detector (DAD). The compounds were separated on a Prodigy column (ODS 3, 150 x 3 mm, 5 µm, 100 Å; Phenomenex,

Aschaffenburg, Germany) with a security guard C18 (ODS 3, 4 x 3 mm, 5 µm, 100 Å) at 30 °C using a water/ acetonitrile (ACN) gradient. Solvent A consisted of 99.5% water and 0.5% acetic acid (Merck, Darmstadt, Germany) whereas solvent B was 100% acetonitrile (J.T. Baker, Deventer, The Netherlands). Separate gradients were used for flavonol and flavone glycosides as well as caffeic acid derivatives (gradient 1) and anthocyanins (gradient 2). Gradient 1 held the following percentages of ACN: 7 - 9% (10 min), 9 - 12% (20 min), 12 - 15% (55 min), 15 - 50% (5 min), 50% isocratic (5 min), 50 - 7% (5 min), isocratic 7% (3 min). Gradient 2 was distinctly shorter: 10 - 50% B (10 min), 50% B isocratic (10 min), 50 - 10% B (5 min) and 10% B isocratic (5 min). Flow rate of both gradients was 0.4 ml/ min.

Flavonol and flavone glycosides as well as caffeic acid derivatives were detected in the mass spectrometer (MS) as anionic, deprotonated molecular ions and characteristic mass fragment ions using an Agilent series 1100 MSD (ion trap) with electrospray ionization (ESI) as ion source in negative mode. Anthocyanin glycosides were qualified as cations in the positive mode. Nitrogen served as dry gas (10 l/ min; 350 °C) and nebulizer gas (40 psi) to evaporate the solvent and facilitate electrochemical ion production. Helium served as collision gas in the ion trap to provoke fragmentation of the investigated compounds. Mass optimization was performed for the mass-to-charge ratio (*m/z*) of deprotonated quercetin-3-*O*-glucoside ($[M-H]^-$; *m/z* 463).

For identification, mass spectra, retention time (RT), and absorption maxima were compared to standard substances when available or to literature data (DuPont, Mondin, Williamson, & Price, 2000; Llorach et al. 2008). Standard substances were purchased at Carl Roth GmbH (Karlsruhe, Germany; quercetin-3-*O*-glucoside, 5-*O*-caffeoylquinic acid) and Sigma-Aldrich GmbH (Munich, Germany; quercetin-3-*O*-glucuronide, di-*O*-caffeoyltartaric acid, cyanidin-3-*O*-glucoside). DAD was used for quantification, using the detection wavelengths 330 nm (caffeic acid derivatives), 350 nm (flavonol and flavone glycosides) and 520 nm (anthocyanin glycosides). External calibration curves were prepared in the respective relevant concentrations, using the standard substances when available. Cyanidin and quercetin-3-*O*-(6''-*O*-malonyl)-glucosides were quantified as their respective 3-*O*-glucoside equivalents. Caffeoylmalic acid is presented as

5-*O*-caffeoylquinic acid equivalents. Values are given in milligrams per gram DM. Software used for quantification and qualification was ChemStation for LC 3D Rev. B. 01.03 [204], Agilent Technologies 2001 – 2005 and MSD Trap Control 5.3, Build No. 11.0, Bruker Daltonik GmbH, 1998 – 2005, respectively.

3.5 Statistical Analyses

Optimization of the analyses methods for the phenolic compounds were evaluated by two-way ANOVA (Fisher's F-test) and Tukey's Honest Significant Differences (HSD) test with a significance level of $\alpha = 0.05$.

In order to detect significant differences between the radiation treatments in experiment 1, two-way ANOVA was performed (factor 1: radiation treatments, factor 2: growth chambers as block effect) followed by Tukey's HSD test. Each growth chamber was treated as one sample to obtain three true biological replicates. Significance level was $\alpha = 0.05$.

In order to study the relationship between plant ontogeny, radiation intensity, cultivar, and their interactions in experiment 2, multiple regression analysis was performed. Representing plant growth stage, DAP were included to study the influence of ontogeny. Radiation intensity was included as PPFD given in µmol m^{-2} s^{-1}. Based on results of experiment 1, mean PPFD during the light-phase of the last 12 days before harvest was considered to be an important factor influencing flavonoid biosynthesis. The two cultivars entered the analysis as cultivar 1 (red Oak Leaf) and cultivar 2 (red Lollo), respectively. Resulting equations presented here only contain those terms whose influence on the investigated variable significantly differed from zero. Significance level was $\alpha = 0.01$ to correct for the multiple comparisons of multiple regression analysis. Differences regarding growth characteristics were evaluated by two-way ANOVA.

In order to detect significant differences induced by the temperature regimes in experiment 3, two-way ANOVA was performed (factor 1: temperature treatments, factor 2: cultivar) followed by Tukey's HSD test. Two true biological replicates were obtained per cultivar and treatment. Significance level was $\alpha = 0.05$.

Calculations were carried out using Excel 2010 (Microsoft Office, Microsoft Corp., Redmond, USA) and STATISTICA (version 10, Statsoft Inc., Tulsa, USA).

4 Results and Discussion

4.1 Identification and quantification of phenolic compounds in lettuce

In order to study the influence of radiation, temperature and growth stage on red leaf lettuce, a reliable analysis method had to be established. The following chapters document how methods for flavonoid glycoside and caffeic acid derivatives analysis which had been described in the literature were adjusted, optimized as well as successfully evaluated to be reliable for the major phenolic compounds of red Oak Leaf and red Lollo lettuce via HPLC-DAD-ESI-MS^n.

4.1.1 Flavonol and flavone glycosides as well as caffeic acid derivatives

4.1.1.1 Identification via mass spectrometry

Ion trap mass spectrometry can provide very detailed information on the analyzed compounds: Ions constituting the base peak in the MS spectrum (peak with highest intensity) are retained in the collision cell by the combination of a magnetic and an electric field where they collide with helium molecules. As a result, kinetic energy is transferred upon the analytes, putting them in an unstable state. Stability is regained by dissociation of the weakest bonds producing characteristic fragments. The mass to charge ratio of charged fragments will be visible in the MS^2 spectrum. The fragmentation step can be repeated several times with the respective main fragment ion. In the negative mode, anions will be detected while in the positive mode only cations are measured.

For identification, MS spectra were compared to standard substances when available and to data published by Llorach et al. (2008). Flavonol and flavone glycosides as well as caffeic acid derivatives were detected in the negative mode because formation of anions by hydrogen ion abstraction was possible (by electrospray ionization) and the level of background signals is lower in negative mode. Detected deprotonated molecule ions and fragment ions (fig. 11, 13, 15, and 17) correspond well to those in the literature. Major flavonol and flavone glycosides as well as caffeic acid derivatives were quercetin-3-*O*-(6''-*O*-malonyl)-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide, chicoric acid (di-*O*-caffeoyltartaric acid), chlorogenic acid (5-*O*-caffeoylquinic acid), and caffeoylmalic acid.

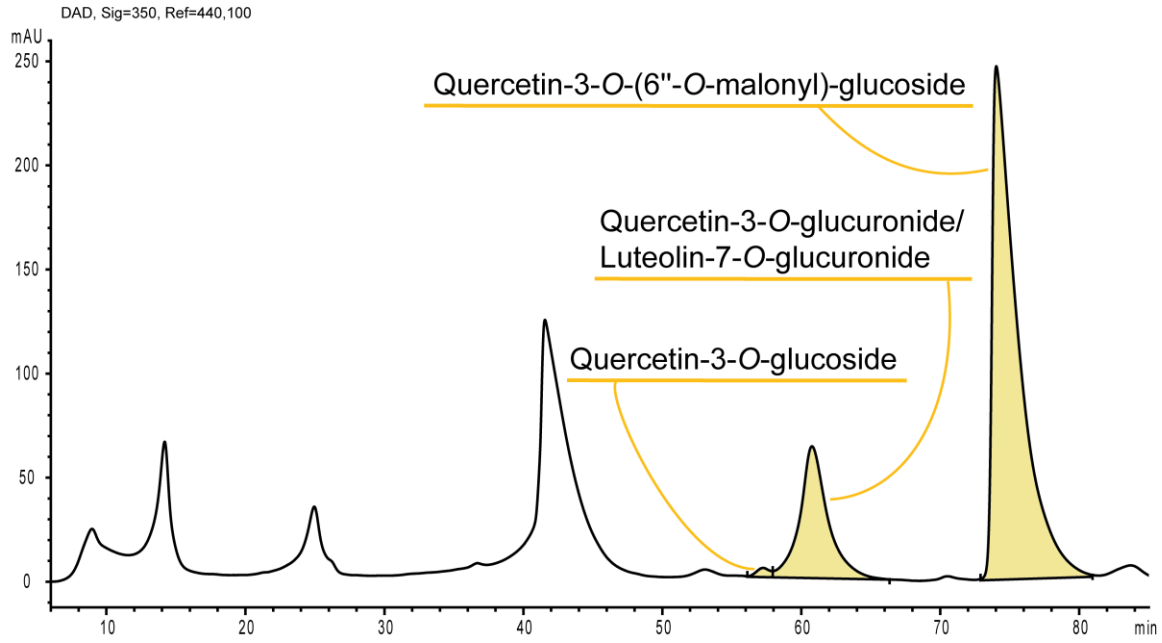

Figure 10: DAD chromatogram of red leaf lettuce extract, prepared with 50% aquaeous methanol, separated by HPLC, detected at 350 nm. Integrated peaks are quercetin-3-*O*-glucoside at a retention time (RT) of 57.3 minutes, quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide (co-eluting) at RT 60.8 min, and quercetin-3-*O*-(6''-*O*-malonyl)-glucoside at RT 74.0 min.

Quercetin and luteolin glycosides were monitored at 350 nm by the diode array detector (fig. 10). DAD absorption spectra of the respective peaks (see appendix, p.118-119, fig. 41, 42, and 43) correspond well to quercetin glycosides. All absorption maxima lie in the UV wavebands (approximately 220, 260, and 350 nm). For quantification, the 350 nm maxima were used. Fig. 43 shows the absorption spectrum of quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide which co-eluted. Molecular ions and characteristic fragment ions for both substances were detected via MS (fig. 15 and 17). Mass spectrometric data (evaluation of extracted ion chromatograms provided by the ion trap MS; data not shown) suggested they contribute to the peak in equal shares which is in line with the literature (DuPont et al. 2000). Structures of luteolin and quercetin resemble each other very much and are therefore very difficult to separate. Also Llorach et al. (2008) did not achieve a separation of these glycosides in extracts of red Oak Leaf lettuce and red Lollo but reported quercetin-3-*O*-glucuronide, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside and the three respective luteolin glycosides to co-elute. In the analyses presented here, no flavonoid rutinosides were detected. This may be due to differences between varieties (DuPont et al. 2000).

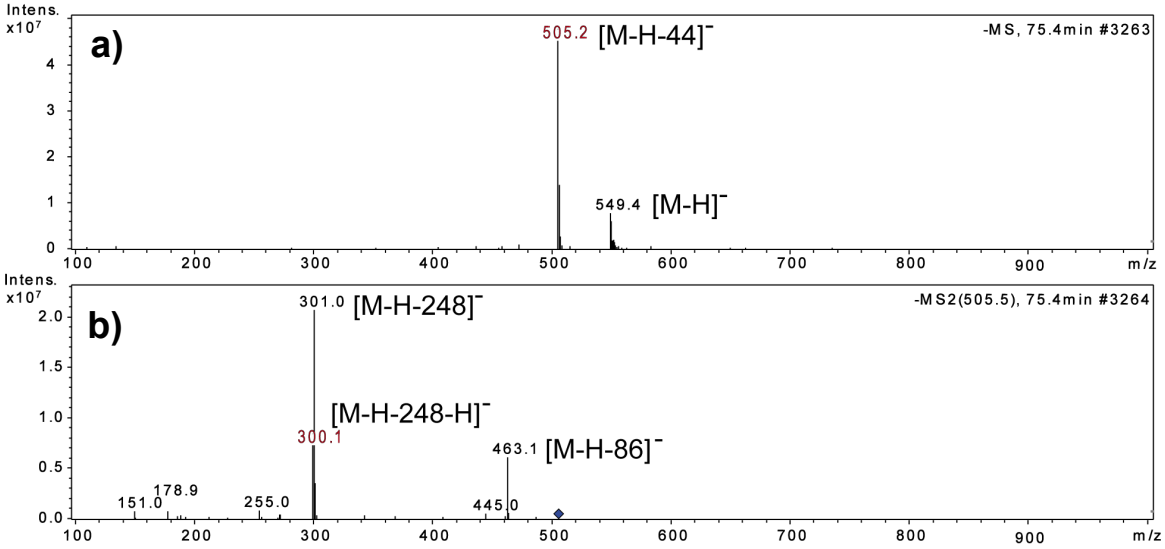

Figure 11: Mass spectrometric data for quercetin-3-*O*-(6''-*O*-malonyl)-glucoside. The deprotonated molecular ion (*m/z* 549) is not the main signal in the MS spectrum (**a**) but the putatively decarboxylized molecular ion (*m/z* 505). In MS² (**b**), fragments of the latter one are visible: *m/z* 463, and *m/z* 301 and 300.

In fig. 11 a and b, the MS and MS² spectra of quercetin-3-*O*-(6''-*O*-malonyl)-glucoside are displayed. The detected mass to charge ratios are further explained by the putative fragmentation patterns in fig. 12 a – d which are compiled based on information given by Llorach et al. (2008).

Generally, flavonoid glycosides often fragment into the aglycone and the sugar moiety as both products are quite stable on their own. Fragmentation steps leading to stable products happen easily (Vollhardt and Schore 2007).

Interestingly, the base peak in fig. 11 a does not correspond to the deprotonated molecular ion shown in fig. 12 a as was expected. The peak with the highest intensity ([M-H-44]⁻) has a lower mass-to-charge ratio, probably a product of insource fragmentation in the ESI. In a similar situation, Llorach et al. (2008) suggested the ion to be the decarboxylized molecular ion after decarboxylation at the malonyl moiety. Therefore, the structure shown in fig 12 b is suggested to constitute the base peak of *m/z* 505. The other fragment ions visible in the MS² spectrum (fig 11 b) are suggested to be the demalonized fragment ion (quercetin-3-*O*-glucoside; fig. 12 c) and the quercetin aglycone (fig. 12 d) where the whole glycosidic moiety has been lost.

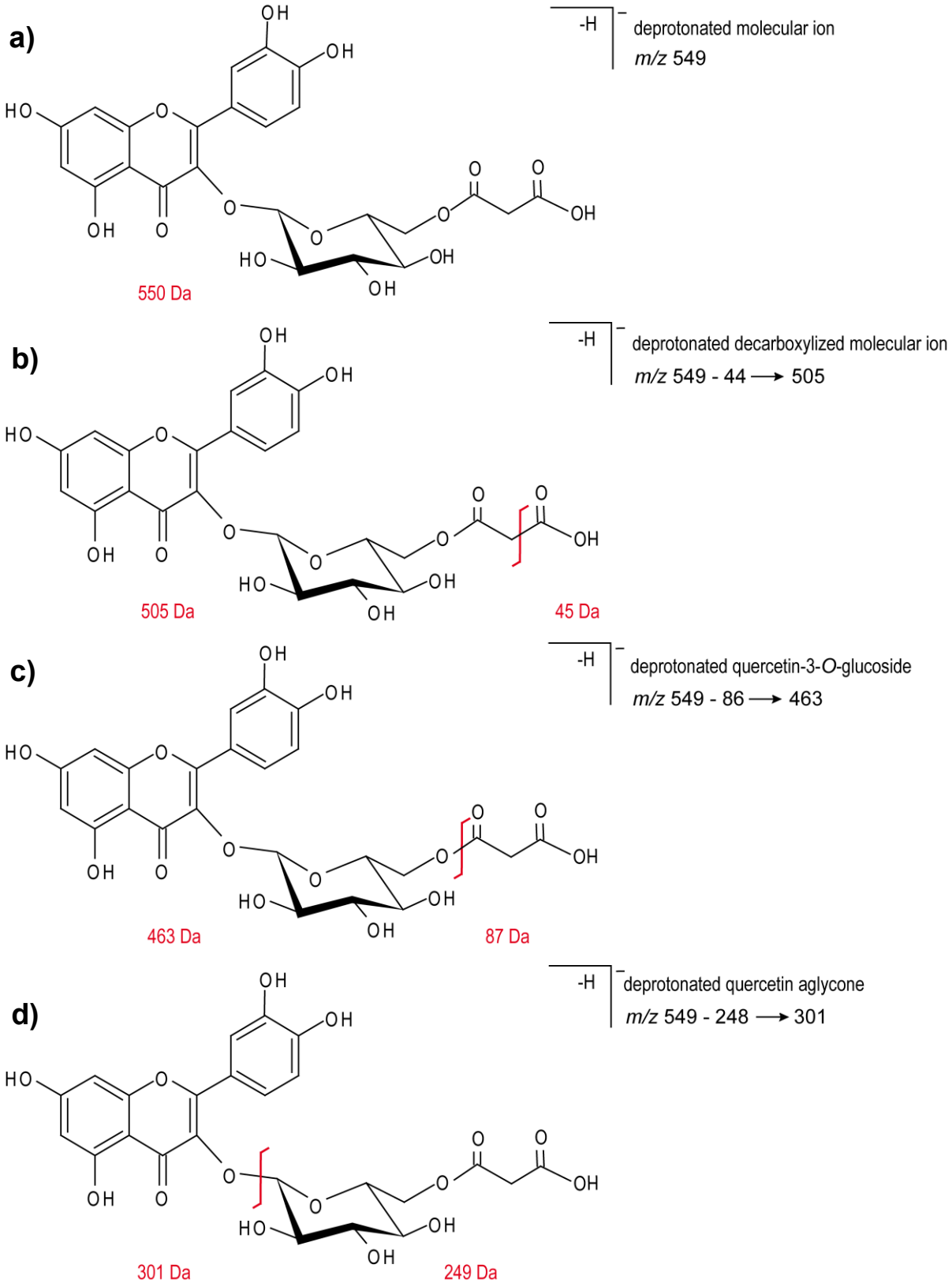

Figure 12: Putative fragmentation pattern for quercetin-3-*O*-(6''-*O*-malonyl)-glucoside, from the molecular ion (**a**) to the decarboxylized (**b**), demalonized (**c**) and completely deglycolized (**d**) fragment ions. The red line indicates the dissociated bond.

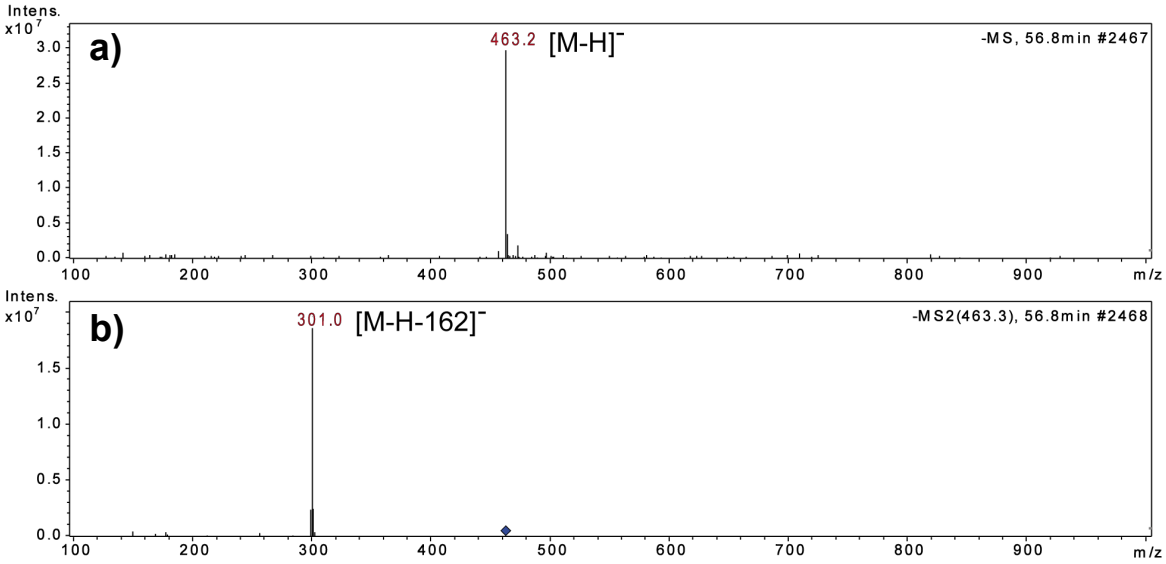

Figure 13: Mass spectrometric data for quercetin-3-*O*-glucoside. The deprotonated molecular ion (*m/z* 463) in the MS spectrum (**a**) gave way to the fragment ion of *m/z* 301 in MS² (**b**). Retention time: 56.8 minutes.

The MS and MS² spectra of quercetin-3-*O*-glucoside (fig. 13 a and b) suggest the fragmentation pattern presented in fig. 14, assigning the *m/z* 463 peak to the deprotonated molecular ion (fig. 14 a) and the *m/z* 301 peak to the deprotonated quercetin aglycone (fig. 14 b).

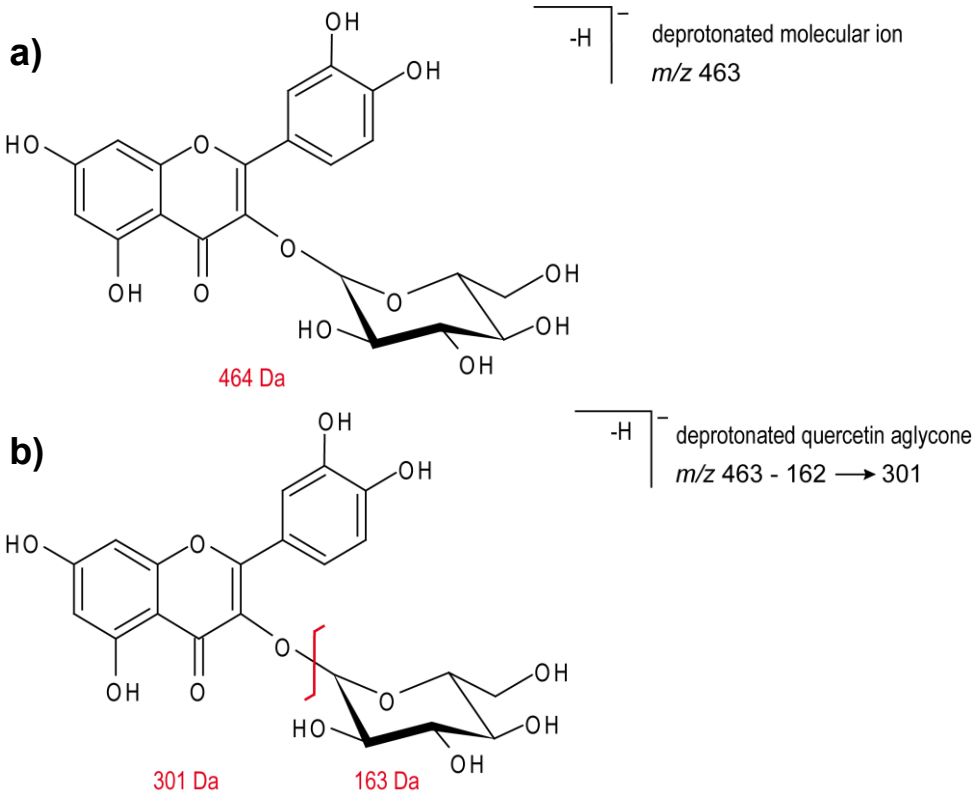

Figure 14: Putative fragmentation pattern for quercetin-3-*O*-glucoside: the molecular ion (**a**) and the deglycolized fragment ion (**b**). The red line indicates the dissociated bond.

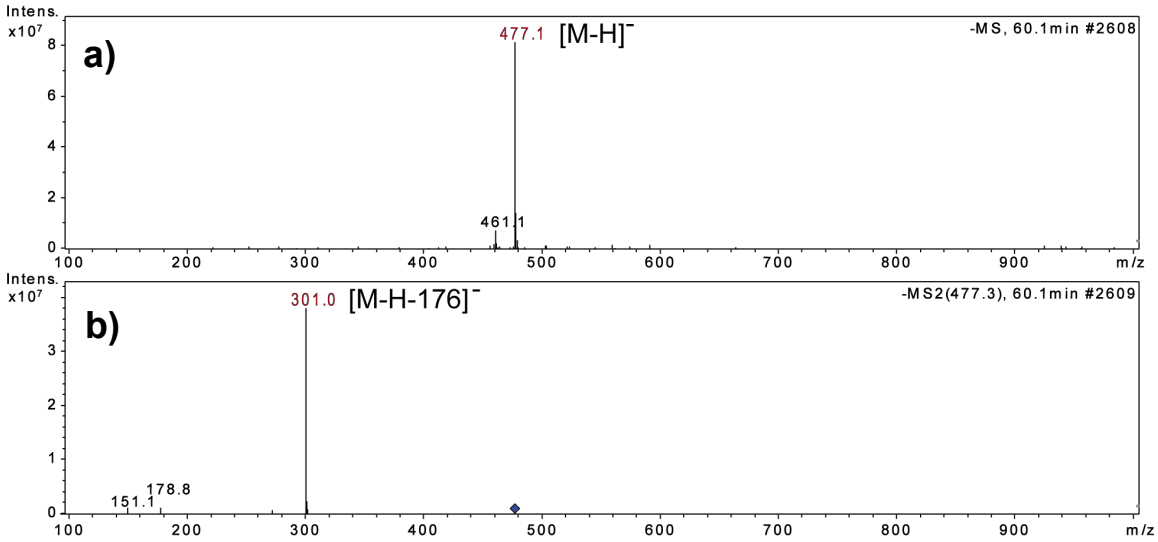

Figure 15: Mass spectrometric data for quercetin-3-*O*-glucuronide. The deprotonated molecular ion (*m/z* 477) in the MS spectrum (**a**) gave way to the fragment ion of *m/z* 301 in MS² (**b**). Retention time: 60.1 minutes.

The MS and MS² spectra of quercetin-3-*O*-glucuronide (fig. 15 a and b) suggest the fragmentation pattern presented in fig. 16, assigning the *m/z* 477 peak to the deprotonated molecular ion (fig. 16 a) and the *m/z* 301 peak to the deprotonated quercetin aglycone (fig. 16 b).

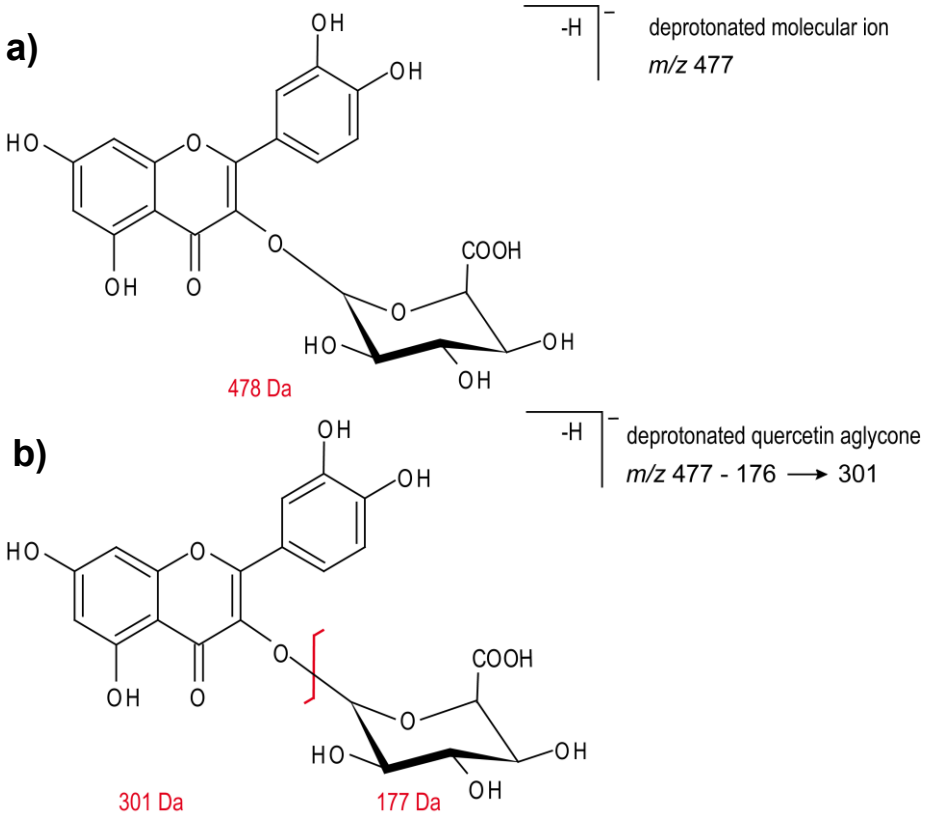

Figure 16: Putative fragmentation pattern for quercetin-3-*O*-glucuronide: molecular ion (**a**) and the deglycolized fragment ion (**b**). The red line indicates the dissociated bond.

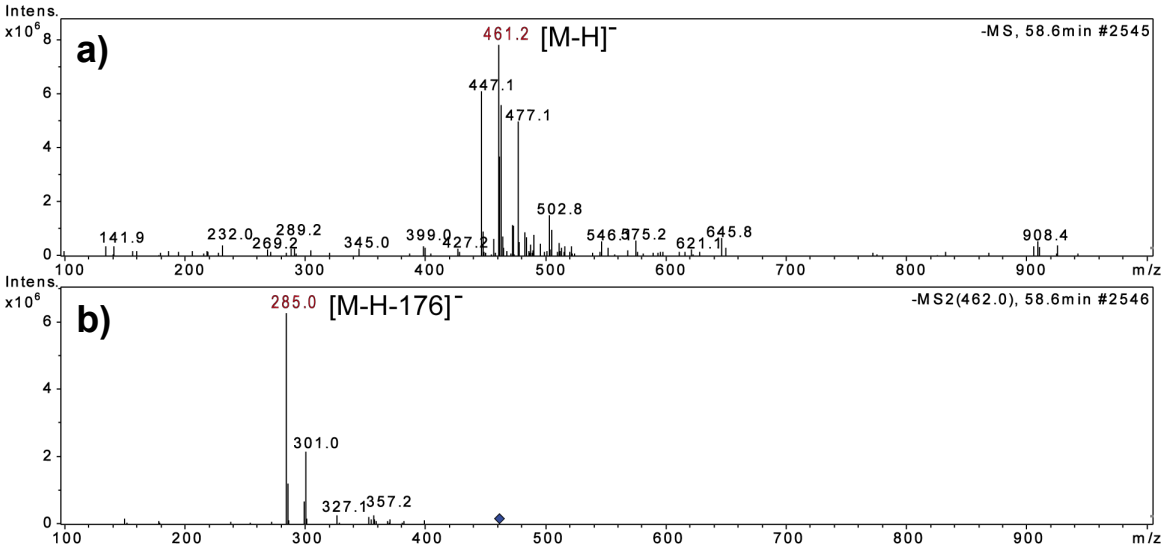

Figure 17: Mass spectrometric data for luteolin-3-*O*-glucuronide. The deprotonated molecular ion (*m/z* 461) visible in the MS spectrum (**a**) gave way to the fragment ion of *m/z* 285, in MS² (**b**). Retention time: 58.6 minutes. These spectra demonstrate that and luteolin-7-*O*-glucuronide (*m/z* 461), luteolin-7-O-glucoside (*m/z* 447), and quercetin-3-*O*-glucuronide (*m/z* 477) co-eluted.

The MS and MS² spectra of luteolin-3-*O*-glucuronide (fig. 17 a and b) suggest the fragmentation pattern presented in fig. 18, assigning the *m/z* 461 peak to the deprotonated molecular ion (fig. 18 a) and the *m/z* 285 peak to the deprotonated luteolin aglycone (fig. 18 b).

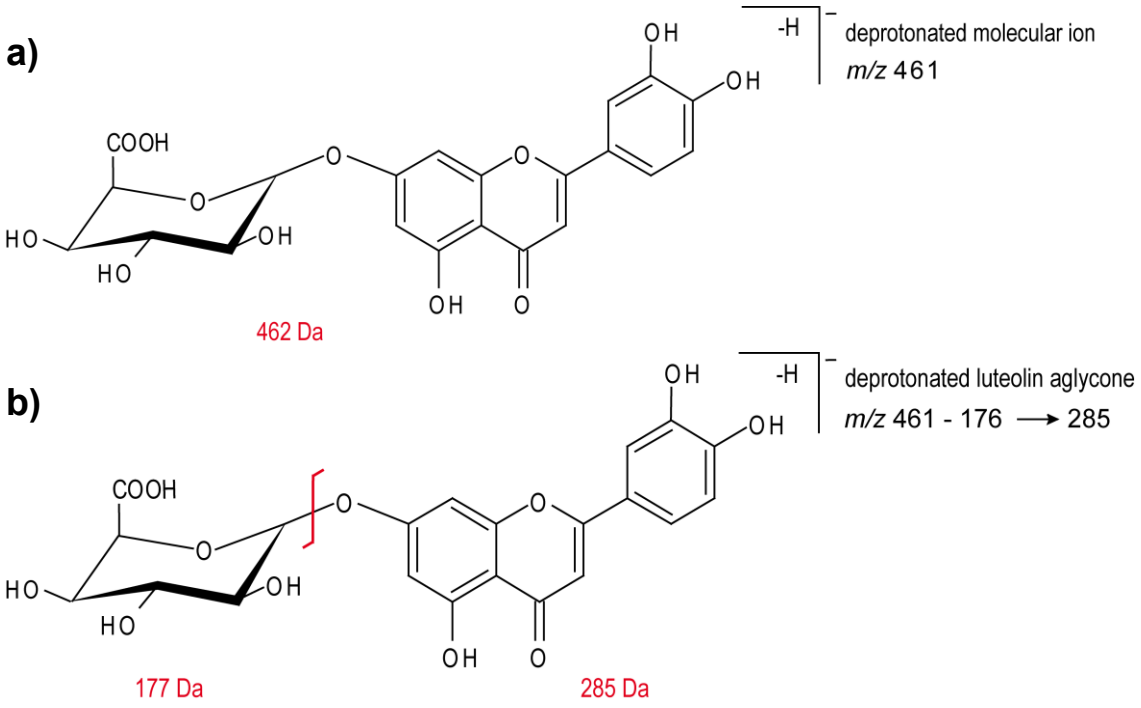

Figure 18: Putative fragmentation pattern for luteolin-3-*O*-glucuronide: the molecular ion (**a**) and the deglycolized fragment ion (**b**). The red line indicates the dissociated bond.

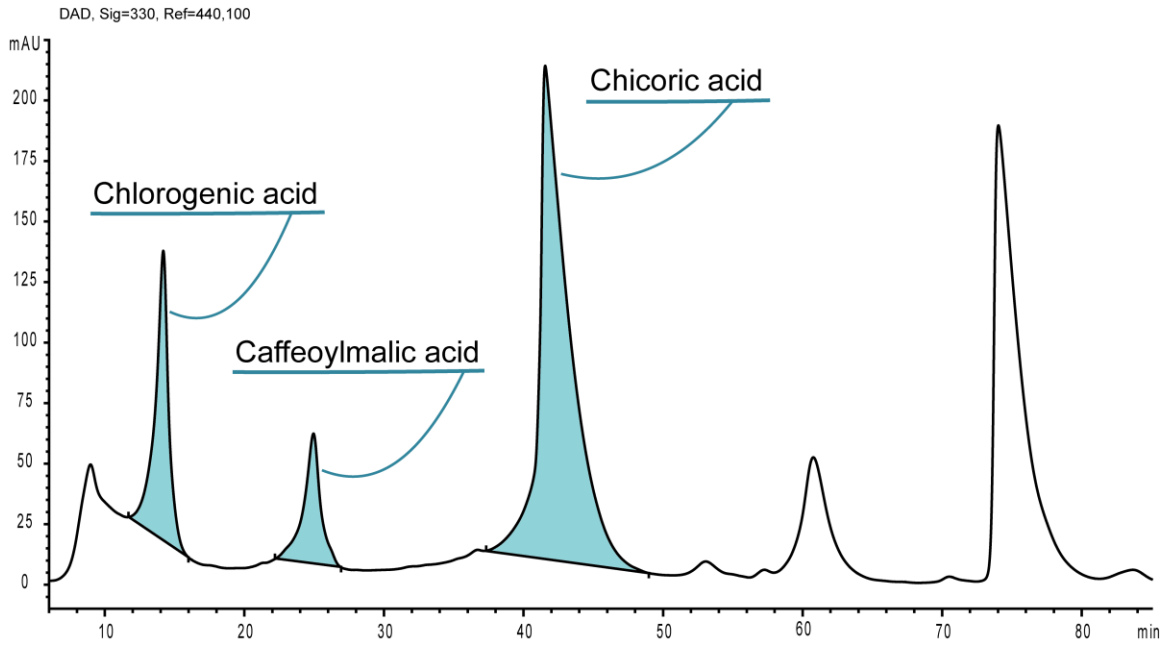

Figure 19: DAD chromatogram of red leaf lettuce extract, prepared with 50% aquaeous methanol, separated by HPLC, detected at 330 nm. Integrated peaks are chlorogenic acid (5-*O*-caffeoylquinic acid; RT 14.2 min), caffeoylmalic acid (RT 25.0 min), and chicoric acid (di-*O*-caffeoyltartaric acid; RT 41.6 min).

Caffeic acid derivatives were monitored at 330 nm by the diode array detector (fig. 19). DAD absorption spectra of the respective peaks (see appendix, p.119-120, fig. 44, 45, and 46) correspond well to caffeic acid derivatives. All absorption maxima lie in the UV wavebands (220, 330 nm). The 330 nm maximum was used for quantification.

For identification, MS spectra were compared to standard substances when available and to data published by Llorach et al. (2008) and Chen et al. (2012). Deprotonated molecule ions and fragment ions detected in the chicoric, chlorogenic, and caffeoylmalic acid peaks (Fig. 20, 22, and 24) correspond well to data in the literature.

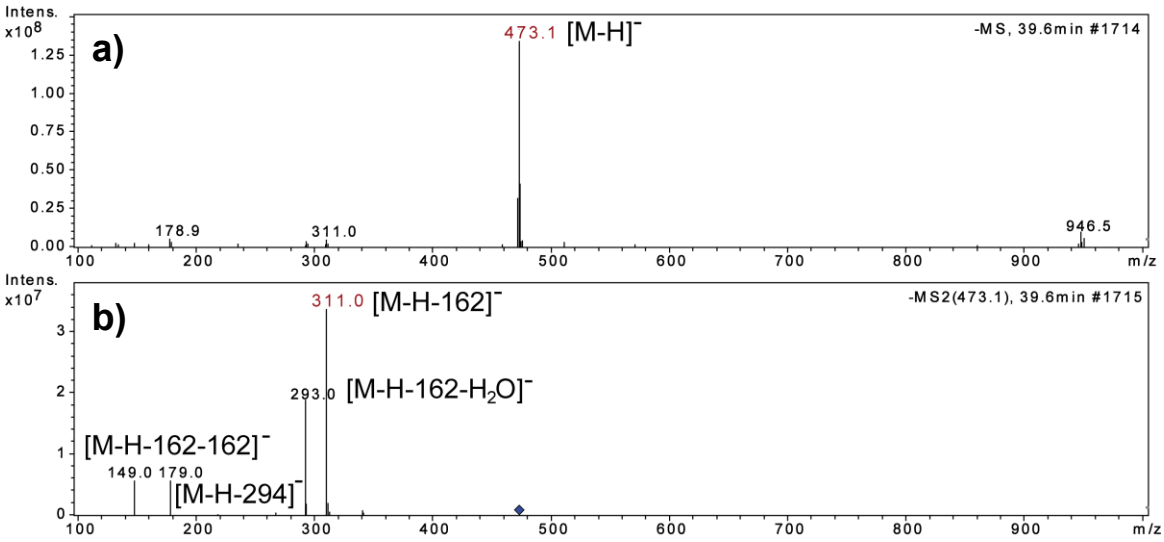

Figure 20: Mass spectrometric data for chicoric acid. The deprotonated molecular ion (*m/z* 473) in the MS spectrum (**a**) gave way to the fragment ions of *m/z* 311, *m/z* 293, *m/z* 179, and *m/z* 149 in MS² (**b**).

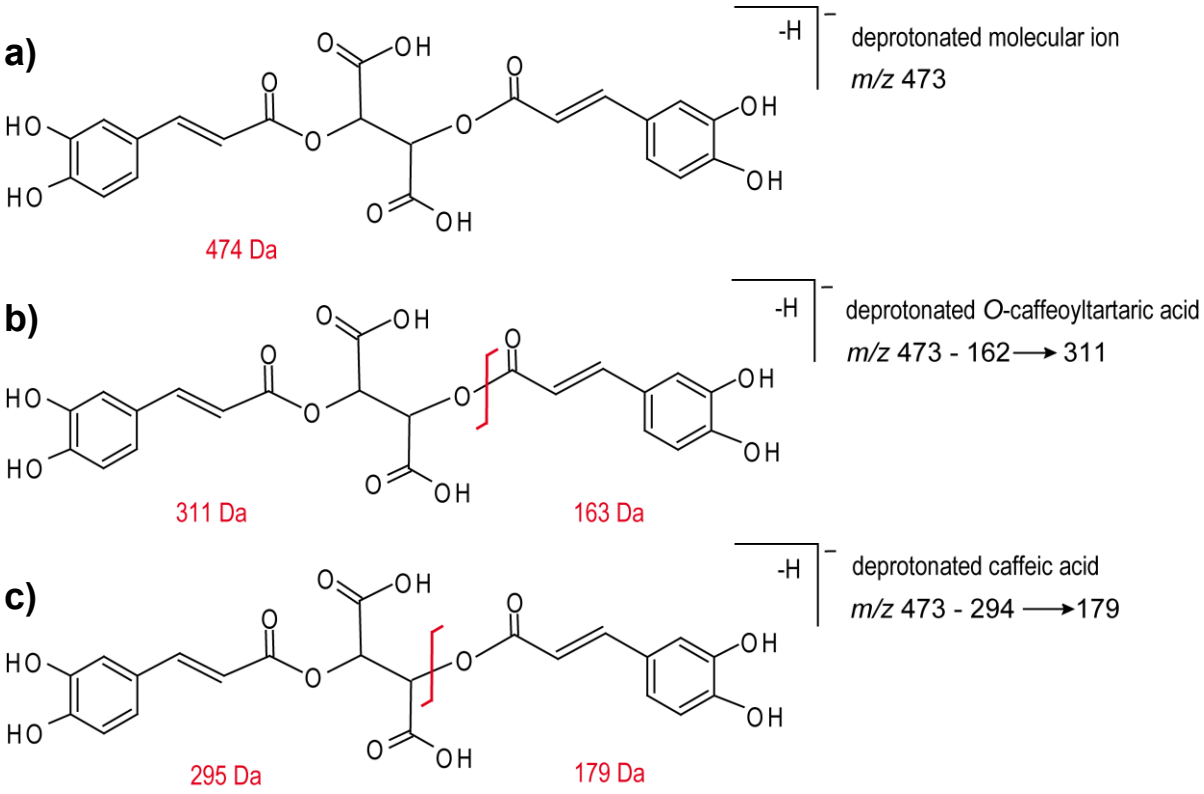

Figure 21: Putative fragmentation pattern for chicoric acid: the molecular ion (**a**),*O*-caffeoyltartaric acid (**b**), and caffeic acid (**c**). The red line indicates the dissociated bond.

The MS and MS² spectra of chicoric acid (fig. 20 a and b) suggest the fragmentation pattern presented in fig. 21, assigning the *m/z* 473 peak to the deprotonated molecular ion (fig. 21 a) and the *m/z* 311 peak to the fragment ion which may be the product of an α-cleavage (fig. 21 b). In an α-cleavage the bond in α-position (the closest one) to the carbonyl C atom dissociates (Vollhardt and Schore 2007) because of the differential electronegativity, i.e. the ability of carbon and oxygen to attract electrons. Hence, the σ-electrons forming the α-bond are not distributed homogenously, weakening the bond and making it prone to cleavage. Two small peaks of fragment ions are visible in MS² (fig. 20 b). The one with *m/z* 149 corresponds to the tartaric acid moiety after the loss of both caffeic acid moieties (potentially in continuation of the mechanism suggested in fig. 21 b). The other one with *m/z* 179 may have been produced by a McLafferty rearrangement resulting in β-cleavage (the dissociation of the next but one-bond to the carbonyl group) as indicated in fig. 21 c. McLafferty rearrangements can occur when there is a hydrogen atom in γ-position, connected via a sufficiently flexible brigde, which will be relocated to the carbonyl group (Vollhardt and Schore 2007). Llorach et al. (2008) suggest the *m/z* 293 fragment ion being produced from the *m/z* 311 by loss of water. Alternatively, it could result from the 294 Da moiety which is lost in the putative McLafferty rearrangement (fig. 21 c) being ionized, e.g. by deprotonation of a carboxyl group. Yet, without further investigation it cannot be determined which (if any of these) mechanism occurs.

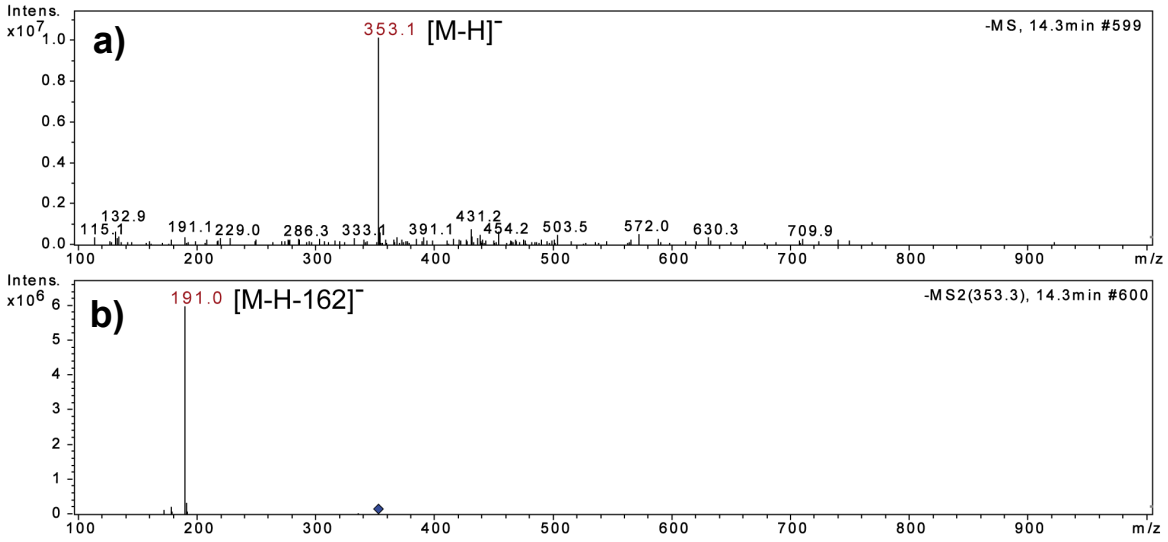

Figure 22: Mass spectrometric data for chlorogenic acid. The deprotonated molecular ion (*m/z* 353) in the MS spectrum (**a**) gave way to the *m/z* 191 fragment ion in MS² (**b**).

The MS and MS² spectra of chlorogenic acid (fig. 22 a and b) suggest the fragmentation pattern presented in fig. 23, assigning the *m/z* 353 peak to the deprotonated molecular ion (fig. 23 a) and the *m/z* 191 peak to deprotonated quinic acid (fig. 23 b) which may have been produced by α-cleavage. Chlorogenic acid does not permit any McLafferty rearrangement as the γ-H atoms are not connected by a flexible bridge.

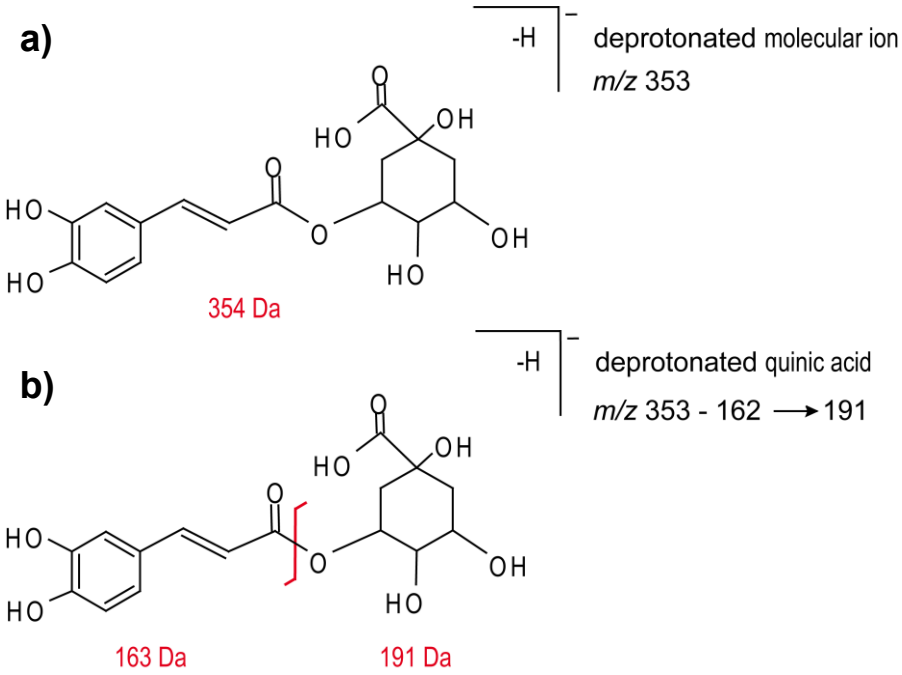

Figure 23: Putative fragmentation pattern for chlorogenic acid: the molecular ion (**a**) and quinic acid (**b**). The red line indicates the dissociated bond.

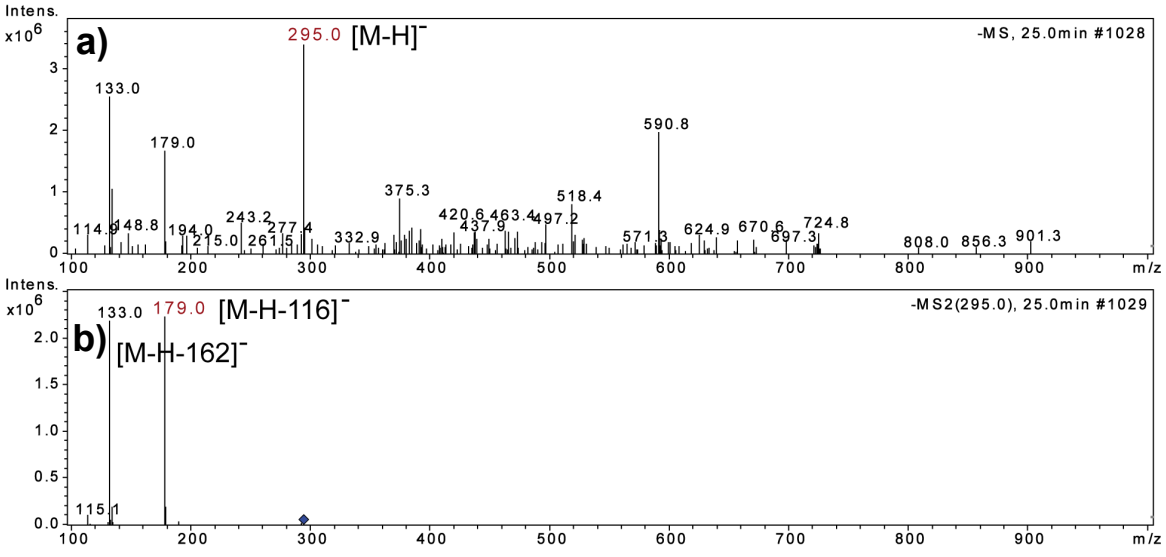

Figure 24: Mass spectrometric data for caffeoylmalic acid. The deprotonated molecular ion (*m/z* 295) in the MS (**a**) produced the *m/z* 179 and *m/z* 133 fragment ions in MS² (**b**).

The MS and MS² spectra of caffeoylmalic acid (fig. 24 a and b) suggest the fragmentation pattern presented in fig. 25, assigning the *m/z* 295 peak to the deprotonated molecular ion (fig. 25 a), the *m/z* 179 peak to deprotonated caffeic acid (fig. 25 b) which may have been produced by McLafferty rearrangement, and deprotonated malic acid (fig. 25 c) which was probably produced by α-cleavage.

The fragment ion with *m/z* 115 showing as a small peak in fig. 24 b may have been produced by the same mechanism responsible for the *m/z* 293 fragment ion in the chicoric acid MS² (fig. 20 b): By ionization of the 116 Da moiety lost in the putative McLafferty rearrangement (fig. 25 c), e.g. by deprotonation of a carboxyl group. Alternatively, the *m/z* 133 fragment ion may have lost a water molecule. Yet, again, the present information only allows for speculation.

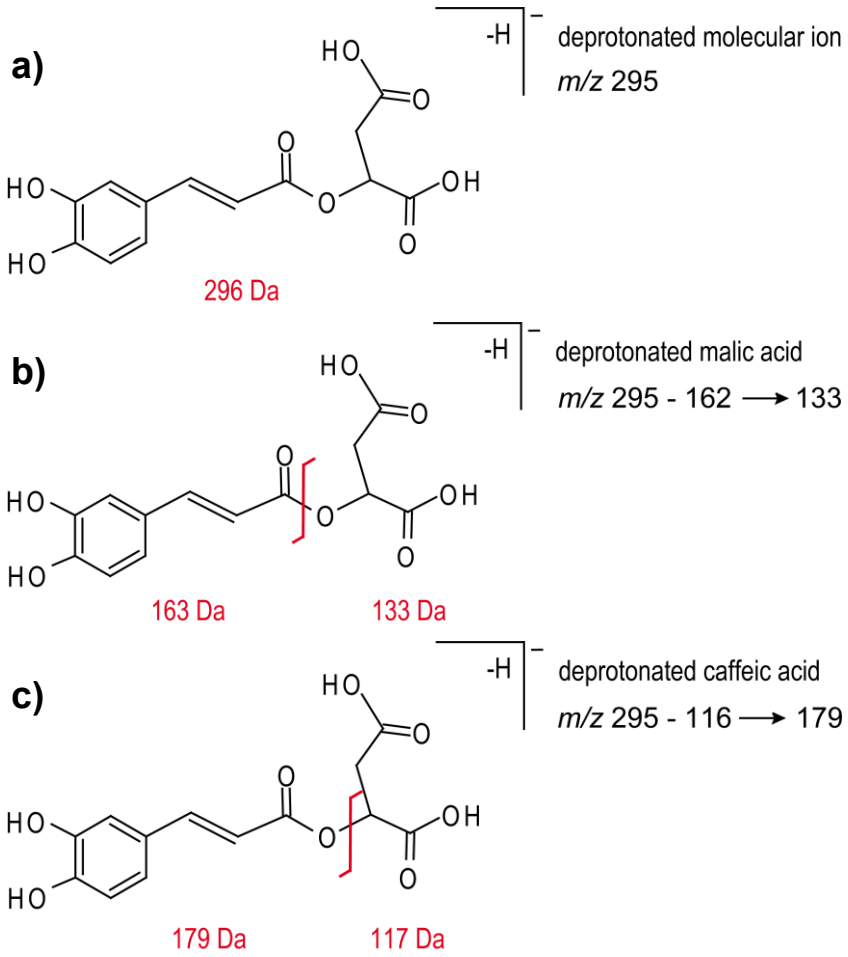

Figure 25: Putative fragmentation pattern for caffeoylmalic acid: the molecular ion (**a**), caffeic acid (**b**), and malic acid (**c**). The red line indicates the dissociated bond.

4.1.1.2 Optimization of sample preparation

The well-established method for analysis of flavonoid glycosides and hydroxycinnamic acids in kale reported by Neugart et al. (2012b) was optimized for lettuce. In duplicate, 0.5 g of the lyophilized and ground plant material were extracted with 25 ml of aquaeous methanol (50% MeOH) for 1, 1.5, 2, 4, and 6 hours, respectively. Comparison of the yielded DAD peak areas revealed that extraction from 1 to 4 hours did not lead to significantly different yields (fig. 26). Only after 6 hours, there was a decline. Extraction time of 1.5 hours was chosen because mean yield values were highest and the standard deviation (SD) was lowest.

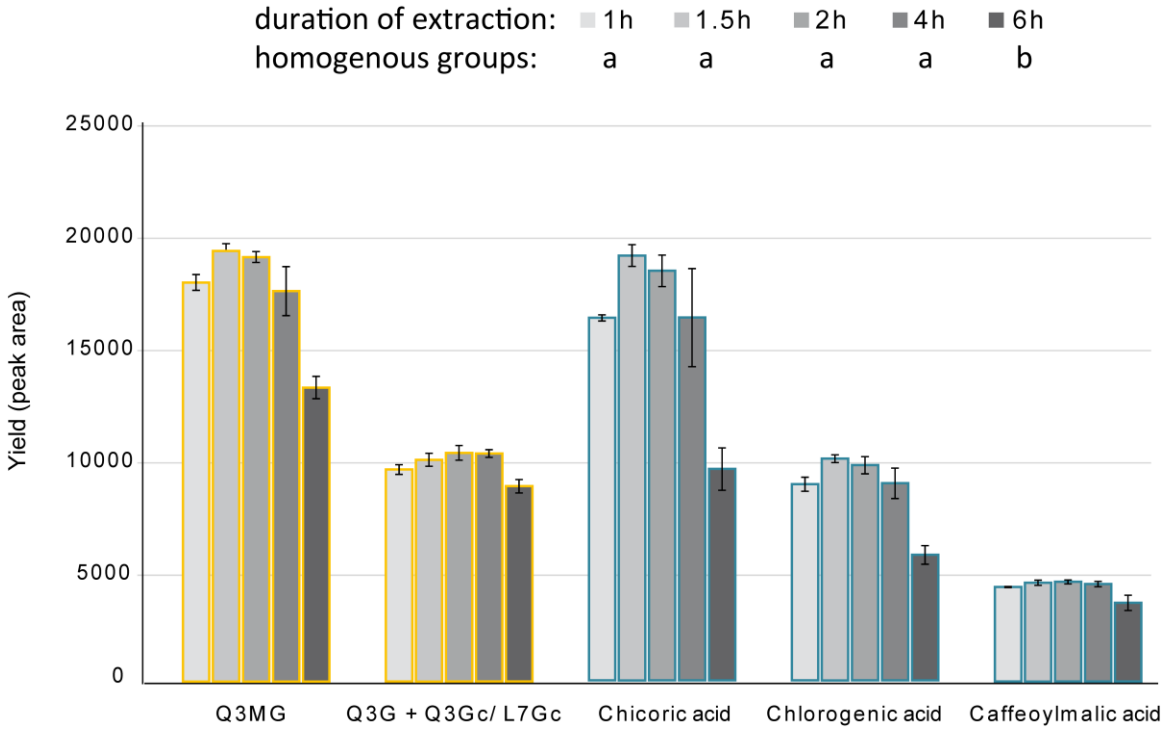

Figure 26: Peak areas yielded for flavonol and flavone glycosides as well as caffeic acid derivatives by extraction with 50% aquaeous methanol for 1, 1.5, 2, 4, and 6 hours, respectively. Q3MG: quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside, Q3G: quercetin-3-*O*-glucosid,Q3Gc/ L7Gc: quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide. Bars representing flavonoid data have a yellow contour while bars representing caffeic acid derivatives have a blue contour. Identical letters below time spans display that, averaged over compounds, yielded peak areas did not differ significantly (mean ± SD; n = 2, Tukey's HSD, α = 0.05).

4.1.1.3 Validation of high performance liquid chromatographic method

Stepwise abridgement of the gradient used by Neugart et al. (2012b) was possible because lettuce contains a smaller number of phenolic compounds than kale. The duration of the gradient could be reduced from originally 175 to 103 minutes. Eluent A was 99.5% water and 0.5% acetic acid, eluent B was 100% acetonitrile. The gradient held the following percentages of ACN: 7 - 9% (10 min), 9 - 12% (20 min), 12 - 15% (55 min), 15 - 50% (5 min), 50% isocratic (5 min), 50 - 7% (5 min), isocratic 7% (3 min). Flow rate was 0.4 ml/ min.

The complete analysis method (extraction, separation, and detection) was validated by extracting ten times 0.5 g dry matter from the same sample. Coefficients of variance for quercetin-3-O-(6´´-O-malonyl)-glucoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide were 0.9, 12.8, and 1.7%, respectively, while those for chlorogenic acid, caffeoylmalic acid, and chicoric acid were 3.2, 9.3, and 4.0%, respectively. These values demonstrate that the method is reliable. However, data obtained on caffeoylmalic acid and quercetin-3-*O*-glucoside concentration should be interpreted with care because of the relatively large variance (9.3 and 12.8%, respectively) which was probably due to their relatively low concentration in the sample. Limit of quantification for quercetin-3-*O*-glucoside was 5.1 µg and for caffeoylmalic acid 8.1 µg per gram dry matter.

The HPLC-DAD-ESI-MS^n method part (separation and detection) was validated by measuring the same extract ten times. Coefficients of variance for quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside, quercetin-3-*O*-glucoside, and quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide were 1.5, 10.8, and 0.6%, respectively, while those for chlorogenic acid, caffeoylmalic acid, and chicoric acid were 0.8, 1.2, and 0.5%, respectively. These values confirm the reliability of the method except for quercetin-3-*O*-glucoside where the variance of 10.8% pointed out that caution has to be applied when interpreting.

Recovery rate for chlorogenic acid was 98.4%. It was determined for chlorogenic acid exemplarily because not all compounds were commercially available.

4.1.2 Anthocyanidin glycoside

4.1.2.1 Identification via mass spectrometry

Anthocyanins were monitored at 520 nm by the diode array detector (fig. 27). The absorption spectrum of the peak at 6.9 minutes corresponds well to an anthocyanin (see appendix, p.121, fig. 47). Absorption maxima lie in the UV C, UV B, and green wavebands (220, 280, 520 nm). Although not the highest, the 520 nm maximum is characteristic and unique among the extracted compounds and was therefore used for quantification.

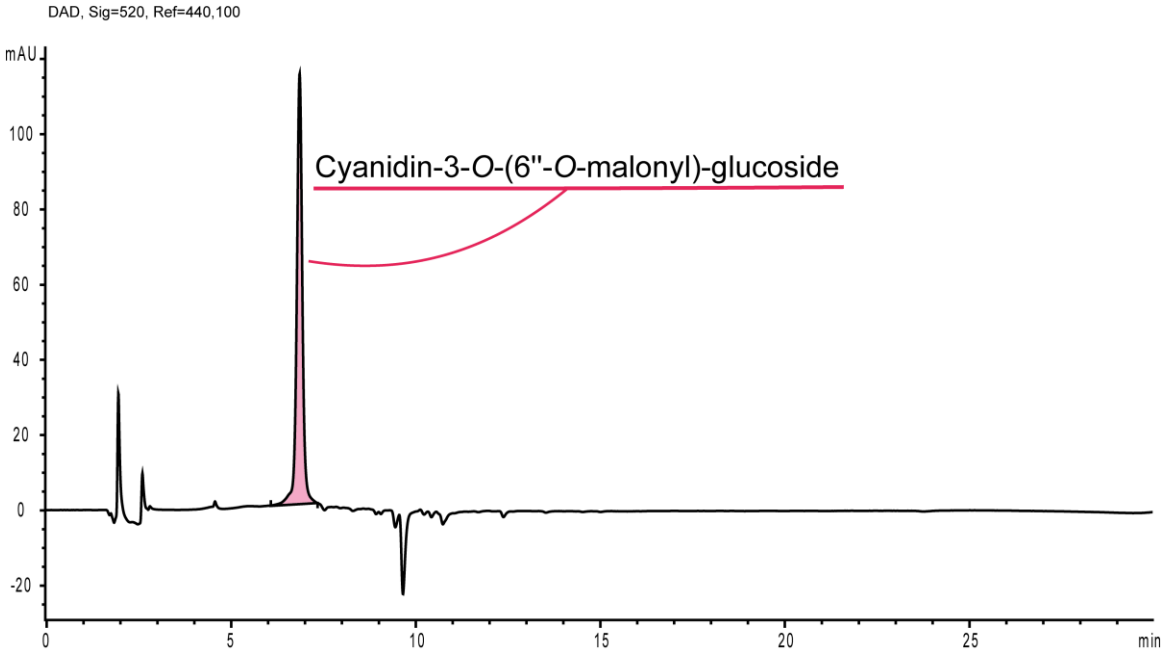

Figure 27: DAD chromatogram of red leaf lettuce extract, prepared with acidified aquaeous methanol (40% MeOH, 10% acetic acid, 50% H_2O) separated by HPLC, detected at 520 nm. The integrated peak is cyanidin-3-*O*-(6''-*O*-malonyl)-glucoside (RT: 6.9 min).

For identification, MS spectra were compared to standard substances when available and to data published by Llorach et al. (2008). Unlike flavonol and flavone glycosides as well as caffeic acid derivatives, anthocyanin glycosides were detected in the positive mode of the MS because they comprise a positive charge in the C ring (oxonium ion).

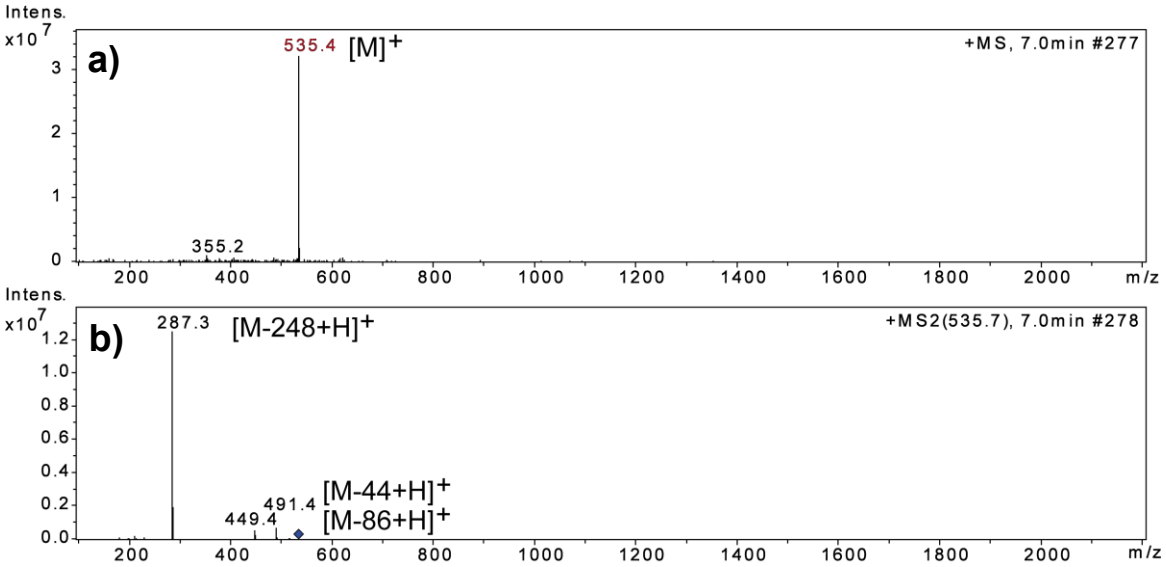

Figure 28: Mass spectrometric data for cyanidin-3-*O*-(6''-*O*-malonyl)-glucoside. The molecular ion (*m/z* 535) in the MS spectrum (**a**) fragmented into *m/z* 287, *m/z* 491, and *m/z* 449 in MS^2 (**b**).

The base peak with a mass-to-charge ratio of *m/z* 535 (fig. 28 a) and retention time of 6.9 minutes did not correspond to cyanidin-3-*O*-glucoside when compared to the standard substance (data not shown). It did, however, correspond very well to the cyanidin-3-*O*-(6''-*O*-malonyl)-glucoside ion reported by Llorach et al. (2008). The main fragment ion (*m/z* 287) in fig. 28 b corresponds well to the protonated cyanidin aglycone (fig. 29 d) while the other two minor fragment ions (*m/z* 491 and *m/z* 449) correspond to the decarboxylized, protonated form of the molecular ion and protonated cyanidin-3-O-glucoside (fig. 29 b and c)

Detection of cyanidin-3-*O*-glucoside in red leaf lettuce in other publications (Wu and Prior 2005a) may be due to differences between cultivars or due to extraction conditions: Demalonation happens easily during the process, especially if inorganic acids are used for acidification (Yamaguchi et al. 1996).

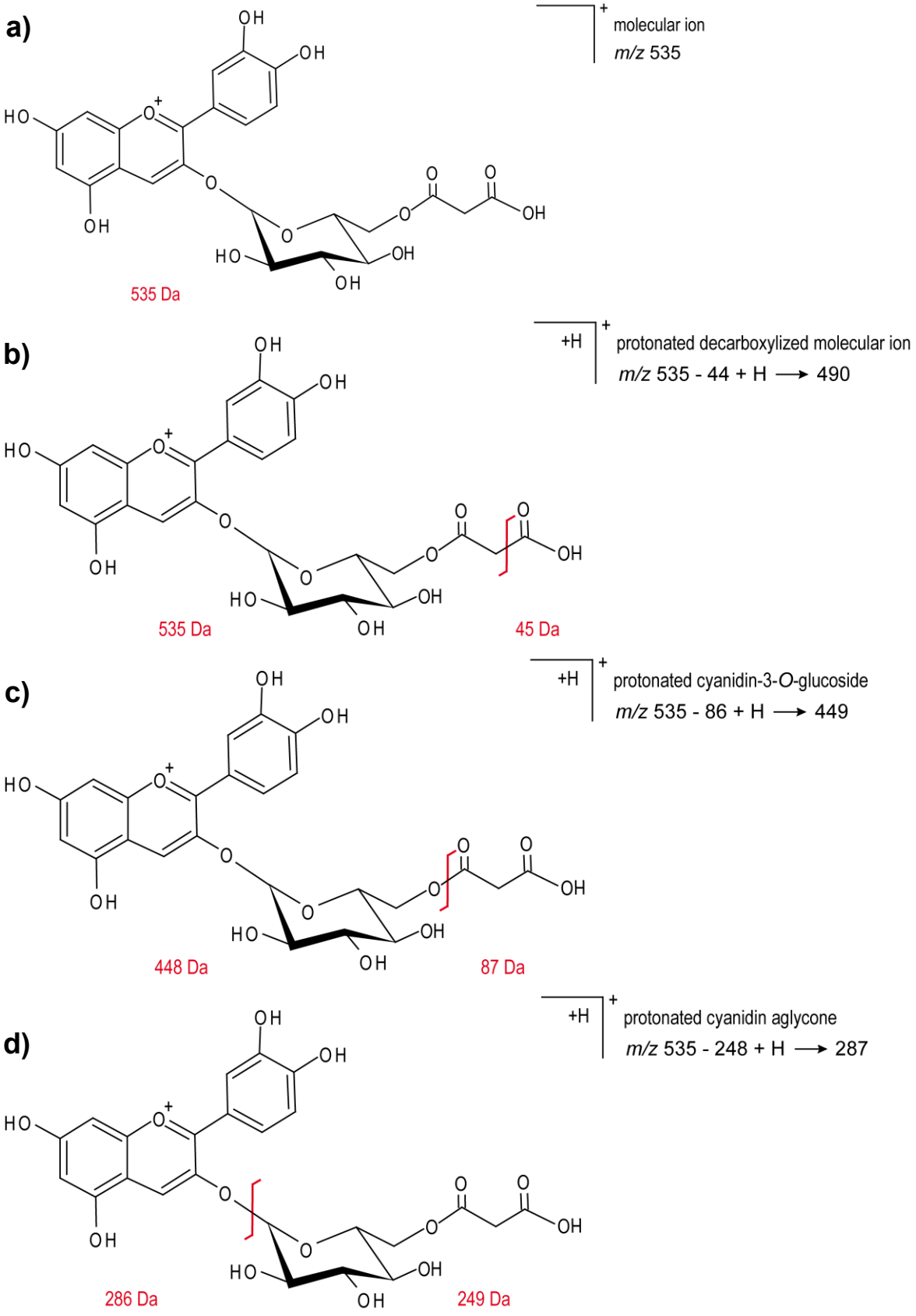

Figure 29: Putative fragmentation pattern for cyanidin-3-*O*-(6''-*O*-malonyl)-glucoside: the molecular ion (**a**), the decarboxylized protonated molecular ion (**b**), the demalonized, protonated molecular ion (cyanidin-3-*O*-glucoside; **c**), and the protonated cyanidin aglycone (**d**). The red line indicates the dissociated bond.

4.1.2.2 Optimization of sample preparation

In order to find a fast and reproducible method for anthocyanin analysis in red leaf lettuce via HPLC-DAD-ESI-MS^n, four approaches based on different publications were tested. In all approaches, 0.5 g lyophilized, ground dry matter were extracted with 25 ml of 60, 70, and 85% aquaeous methanol, containing 0.5% acetic acid. Approach 1 was based on García-Macías et al. (2007): Suspensions were stored over night in a fridge (dark, 6 °C), then filtered. Approach 2 was based on Wu et al. (2004): Suspensions were places in an ultrasound bath for 5 minutes, then centrifuged. Approach 3 was based on Qian et al. (2007): Suspensions were placed in an ultrasound bath for 60 minutes, then filtered. Approach 4 was based on Neugart et al. (2012a), which was also used for flavonol and flavone glycosides as well as caffeic acid derivatives: Suspensions were stirred for 90 minutes, then filtered.

Highest yields were obtained with approach 4. In comparison, yields increased with higher percentage of water (85 to 60% MeOH). Thus, 50, 40, and 30% MeOH including 0.5% acetic acid were tested, too, with 40% MeOH resulting in the highest yield (fig. 30). During optimization of extraction time, a rapid decline of peak area over time became obvious: From 5 to 360 minutes, peak area declined from 4500 to 100, indicating that the molecule was not stable. Anthocyanins change their color according to pH value due to structural transformations: In acidic pH, the flavylium cation of cyanidin-3-*O*-glucoside displays a red color, turning to the colorless carbinol base in neutral pH (Hatier and Gould 2009). Therefore, the pH of the extraction solvent was lowered from originally 3.2 to 2.6 to establish a pH value in which cyanidin-3-*O*-glucoside is known to be stable (Cabrita et al. 2000). Further investigation showed the anthocyanin red leaf lettuce extracts to be stable at pH 2.6, too: There were no significant differences in yield after extraction for 5 up to 240 minutes with 40% MeOH, 10% acetic acid and 50% water. Yield did not improve when acetic acid concentration was increased to 25% in 40% MeOH (pH 2.1). Interestingly, extraction with 7 or 13% acetic acid in 30 or 50% methanol (both pH 2.5 - 2.6) did not differ either, pointing out the importance of the pH value during extraction.

Although extraction for 5 minutes would have been sufficient, 15 minutes of extraction time were chosen to allow for a robust handling routine, taking the

subsequent step of centrifuging for 15 minutes into account. The extracted plant material was not removed from the extracts by filtering (as suggested by some publications) but by centrifuging because a red rim on the filter paper very obviously stated a loss of substance.

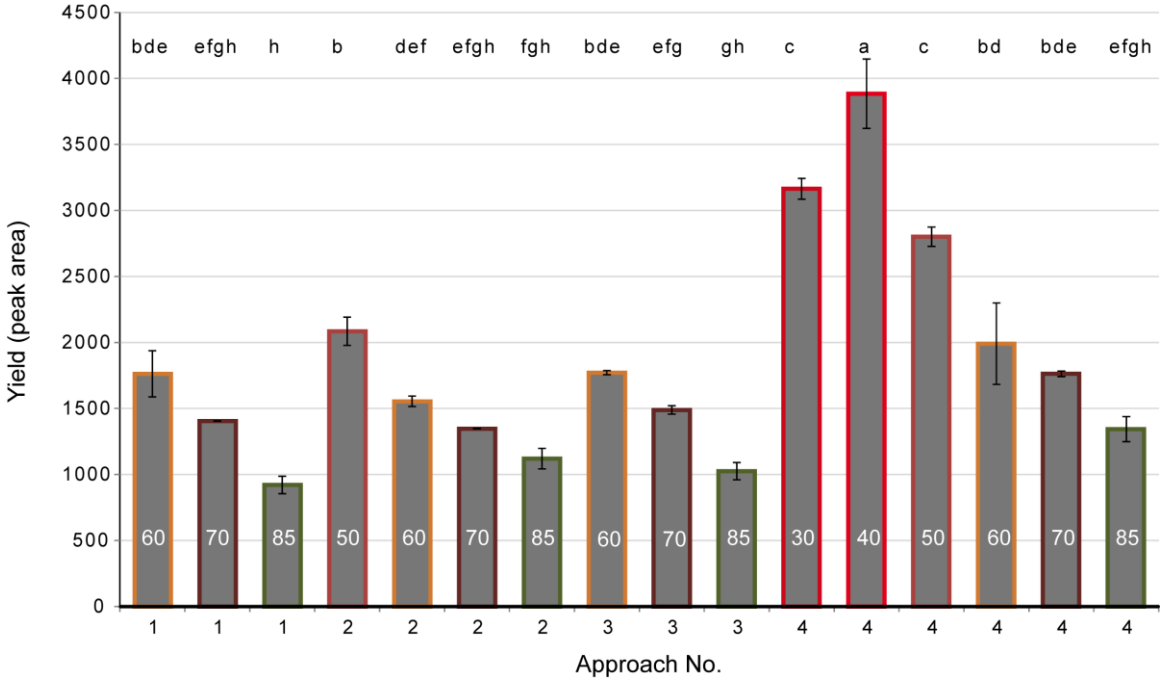

Figure 30: Peak areas yielded with four different approaches to extract anthocyanins from red leaf lettuce, using acidified (0.5% acetic acid) aquaeous methanol. The methanol concentration (30 - 85%) is depicted by white numbers on the bars. The color of the contour displays the color of the respective extract. Identical letters of top of bars display that yielded peak areas did not differ significantly (mean ± SD; Tukey's HSD, $\alpha = 0.05$, $n = 2$)

4.1.1.3 Validation of high performance liquid chromatographic method

Anthocyanin peaks were integrated in the 520 nm DAD chromatogram. The 103 minute gradient used to analyze flavonol and flavone glycosides as well as caffeic acid derivatives could be shortened to 30 minutes because there was only one peak at the relevant wavelength of 520 nm (fig. 27). Eluent A was 99.5% water and with 0.5% acetic acid, eluent B was 100% acetonitrile. The gradient held the following percentages of ACN: 10 - 50% B (10 min), 50% B isocratic (10 min), 50 - 10% B (5 min) and 10% B isocratic (5 min). Flow rate was 0.4 ml/ min.

The complete analysis method (extraction, separation, and detection) was validated by extracting ten times 0.5 g dry matter from the same sample. Coefficient of variance was 1.2%.

The HPLC-DAD-ESI-MS^n method part (separation and detection) was validated by measuring the same extract ten times and produced a coefficient of variance of 1.0%. These values demonstrate that the developed method is very reliable.

4.2 Influence of radiation – Experiment 1: Growth chamber

4.2.1 Plant growth

After 14 days of cultivation in the growth chambers, mean head mass of the unshaded plants did not differ significantly from that of plants growing shaded (tab. 1). However, after 28 days of cultivation, the unshaded lettuce plants had gained a significantly higher mean head mass per plant than the shaded ones (tab. 1). The head mass of the plants that grew first shaded then unshaded did not differ significantly from that of the unshaded plants, but from both the shaded ones and those first unshaded then shaded (tab. 1). Head mass of the latter was significantly lower than head mass of those growing first shaded then unshaded. The results on head mass are in line with results of Sanchez et al. (1989). In their study, they also found that in early growth stages, biomass accumulation is less affected by reduced radiation than in later stages when head formation takes place and most of the biomass is being accumulated.

No significant difference was detected concerning dry matter content of lyophilized samples between the treatments, only between harvest dates. Mean dry matter content of the respective treatments was 8.77 ± 0.84 and 5.89 ± 0.58% after 14 and 28 days of cultivation, respectively.

Table 1: Head mass of red Oak Leaf lettuce cultivated at different PPFD levels for 14 and 28 days after planting, respectively, given in gram fresh matter (FM). Identical letters behind values indicate that these treatments do not differ significantly (mean ± SD; Tukey's HSD-test; α = 0.05, n = 3).

Harvest	unshaded	first shaded then unshaded	first unshaded then shaded	shaded
14 days after planting	79 ± 11 **a**			78 **±** 10 **a**
28 days after planting	290 **±** 38 **ab**	308 **±** 29 **a**	274 **±** 41 **bc**	253 **±** 36 **c**

4.2.2 Flavonoid glycosides

After 28 days of cultivation, a significant influence of the radiation treatment on all glycosides of quercetin and luteolin was detected (fig. 31). Plants growing unshaded all the time displayed a significantly higher concentration of quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside, quercetin-3-*O*-glucuronide/ luteolin-7-O-glucuronide, and quercetin-3-*O*-glucoside than those growing shaded all the time. Interestingly, there were no significant differences between plants growing unshaded only for the last 14 days and those growing unshaded all the time.

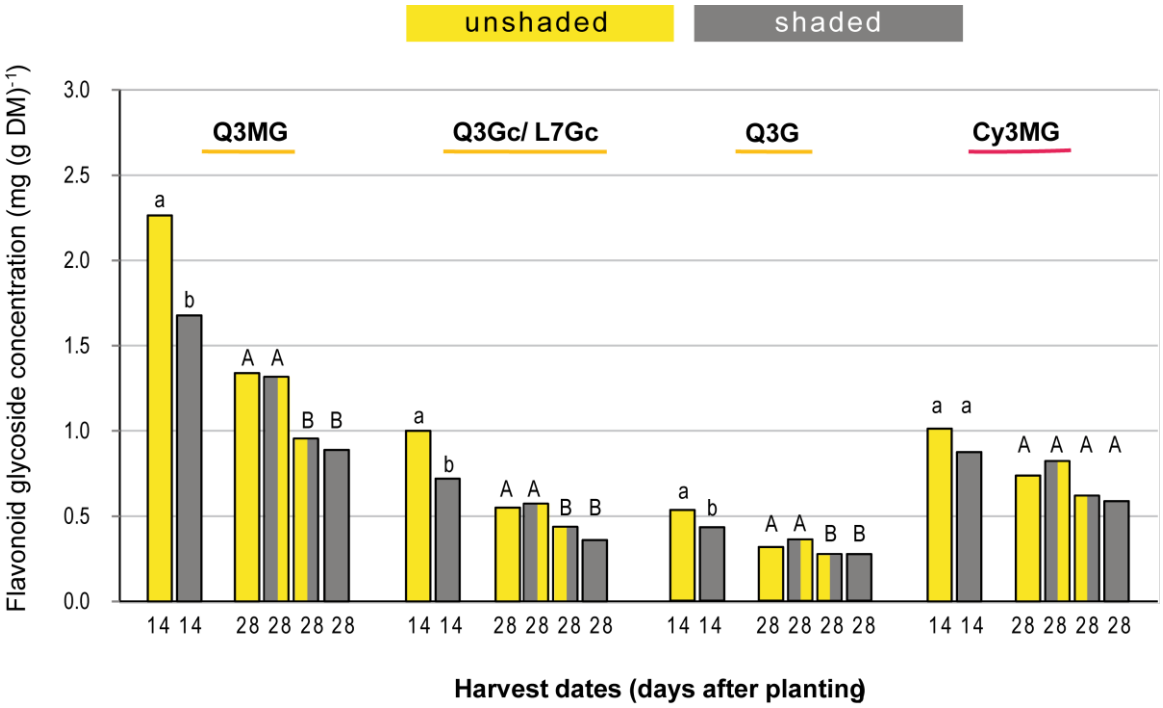

Figure 31: Concentrations of flavonoid glycosides related to dry matter (DM) of red Oak Leaf lettuce, cultivated for 14 and 28 days, respectively, at different light conditions. Yellow bars represent cultivation at 410 µmol m^{-2} s^{-1}, grey bars represent cultivation at 225 µmol m^{-2} s^{-1}. Q3G: quercetin-3-*O*-glucoside, Q3MG: quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside, Q3Gc/ L7Gc: quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide, Cy3MG: cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside. For each compound, identical letters on top of bars show that these treatments do not differ significantly (mean, n = 3; Tukey's HSD test, α = 0.05)

After 14 days of cultivation, the shaded plants already contained lower concentrations of quercetin and luteolin glycosides than their unshaded counterparts (fig. 31). Nevertheless, after being transferred to the unshaded treatment, they were able to fully compensate this deficit until the second harvest date. Consistently, there was no significant difference detectable regarding quercetin and luteolin glycoside concentration of plants that were shaded all the time and those shaded only for the last 14 days.

Higher concentrations of quercetin and luteolin glycosides in the plants exposed to higher PPFD is in agreement with data in the literature (Oh et al. 2009; Agati and Tattini 2010; Wang et al. 2012). After 28 days, the shaded plants had a 34% lower concentration of quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside and of quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide than the unshaded plants as well as a 14% lower concentration of quercetin-3-*O*-glucoside. This decrease is remarkable as it indicates a very close coupling of radiation intensity and *O*-glycosylated flavonol biosynthesis, even at light intensities which were not stressful (Fu et al. 2012).

Even without stress factors, photosynthesis is continuously accompanied by the production of reactive oxygen species and it is crucial for plants to maintain generation and removal of ROS in a finely tuned equilibrium as they also act as signaling molecules (Apel and Hirt 2004). Different stressful factors result in the formation of different, distinct ROS types in plant cells (Edreva 2005a). Singlet oxygen ($^1O_2$) is formed by energy transfer when the chlorophyll molecule of photosystem II is in triplet state due to insufficient energy dissipation during photosynthesis – it is a permanent by-product of photosynthesis but increases drastically in high light intensity (Apel and Hirt 2004). Yet, interestingly, $^1O_2$ is even produced under low-light conditions (Buchert and Forreiter 2010). It has been suggested that in response to different stressors, those flavonoid species may be synthesized which very efficiently scavenge the respective ROS type (Tattini et al. 2004).

Quercetin has a high reaction efficiency towards $^1O_2$ due to the hydroxyl group at C3 position activating the C2-C3 double bond (Tournaire et al. 1993) and might therefore be synthesized by plants to counteract $^1O_2$ formation, possibly in concert with other chloroplast located antioxidants like tocopherol and

carotenoids (Agati et al. 2007). This is in line with the hypothesis recently offered by Agati et al. (2012) suggesting that quercetin protects chloroplasts from $^1O_2$ produced by visible light, emphasizing that chloroplasts are themselves capable of flavonoid synthesis.

Regarding cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside concentration, no significant differences between the plants cultivated under different radiation regimes were detected – neither at the first nor at the second harvest date (fig. 31). Unlike the flavonol and flavone glycosides', the concentration of anthocyanin glycosides was not significantly influenced by the applied PPFD levels. The explanation for the low-key impact on the anthocyanin concentration may be that anthocyanins are known for protecting the photosynthetic apparatus against excess radiation of stressfully high intensities and UV radiation by absorption (Hatier and Gould 2009; Tsormpatsidis et al. 2010). However, only non-stressful 400 µmol m^{-2} s^{-1} (Fu et al. 2012) were applied in the unshaded treatment of the experiment discussed here.

Moreover, the artificial radiation in the growth chambers supplied only low relative proportions of UV radiation: UV A (315 - 380 nm) = 0.7% and UV B (280 - 315 nm) = 0%, respectively, whereas about 80% were in the PAR waveband and 19% near infrared (see appendix, p.123, fig. 48). UV B is considered to be especially biologically active as it is absorbed by DNA molecules and proteins (Meffert and Meffert 2000). Previously published results indicate a strong susceptibility of red leaf lettuce anthocyanin biosynthesis to UV radiation (Tsormpatsidis et al. 2010). The generally non-stressful PPFD and the lack of UV B radiation may explain why no significant impact of radiation intensity was detected in this experiment. The trend observed for the cyanidin glycoside was the same as for the quercetin glycosides. This suggests that something was happening – just not effectively.

Concerning structure, the three measured quercetin glycosides comprise the same aglycone (quercetin) but differ in their sugar moieties (glucoside and glucuronide) and type of acylation (non-acylated and mono-acylated glycosides). The mono-acylated glycoside (quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside) reacted stronger to radiation than the non-acylated glycoside (quercetin-3-*O*-glucoside): Compared to the unshaded plants, the concentration of mono-acylated glycoside

was 34% lower in shaded plants while the concentration of non-acylated glycoside was only 14% lower. Furthermore, the carboxyl group in the glucuronides (quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide) seems to enhance the response to shading in a similar way (34% lower concentration in shaded plants). In addition, two of the investigated flavonoid glycosides comprise the same kind of acylation (malonyl glycoside) but differ in their aglycones (quercetin and cyanidin) resulting in a different response to radiation. This emphasizes the crucial role of the aglycone.

Because of minor or major differences in structure, different flavonoid aglycones and glycosidic moieties, with or without acylation, might prove suitable to counteract different types of oxidative challenges within plants. Structure-antioxidant activity relationships have been reported for non-acylated and acylated flavonol glycosides in kale, differing either in their aglycone structure, their number of glycoside substituents or the kind of acylation (Fiol et al. 2012).

4.2.3 Caffeic acid derivatives

The different radiation conditions had no significant impact on the concentration of any of the three caffeic acid derivatives, neither at the first nor at the second harvest date (fig. 32). This is in line with results published by Agati and Tattini (2010) who also did not find any effect of different radiation treatments (PAR or UV) on hydroxycinnamic acid concentrations in leaves of *Ligustrum vulgare*. Nevertheless, they detected a distinct enhancing effect of radiation, no matter which wavelengths and proportions, on the quercetin concentration. Other studies also showed diverging responses of flavonol glycosides and caffeic acid derivatives to radiation conditions (Oh et al. 2009; Wang et al. 2012).

Chlorogenic acid, chicoric acid and caffeoylmalic acid only absorb radiation in the UV and not in visible wavebands (data derived from DAD spectra, see appendix, p. 119-120, fig. 44, 45, 46) and it has been reported that the caffeic acid derivatives present in lettuce have a lower antioxidant activity than the quercetin and cyanidin derivatives (García-Macías et al. 2007). Thus, their function in the plant might primarily be physical UV protection and not so much chemical radical scavenging and their biosynthesis might be first and foremost triggered by UV radiation and not so much by PAR. Accordingly, García-Macías

et al. (2007) measured increased caffeic acid derivatives concentration in red leaf lettuce exposed to UV radiation compared to plants cultivated under UV blocking films. Hence, the lack of increase of caffeic acid derivatives (that had been expected in reaction to the higher PPFD) in this experiment might also be due to the artificial light in the growth chambers supplying only a small relative proportion of radiation in the UV waveband. The 45% higher UV radiation intensity in the unshaded treatment might still have been too low to enhance caffeic acid derivatives biosynthesis.

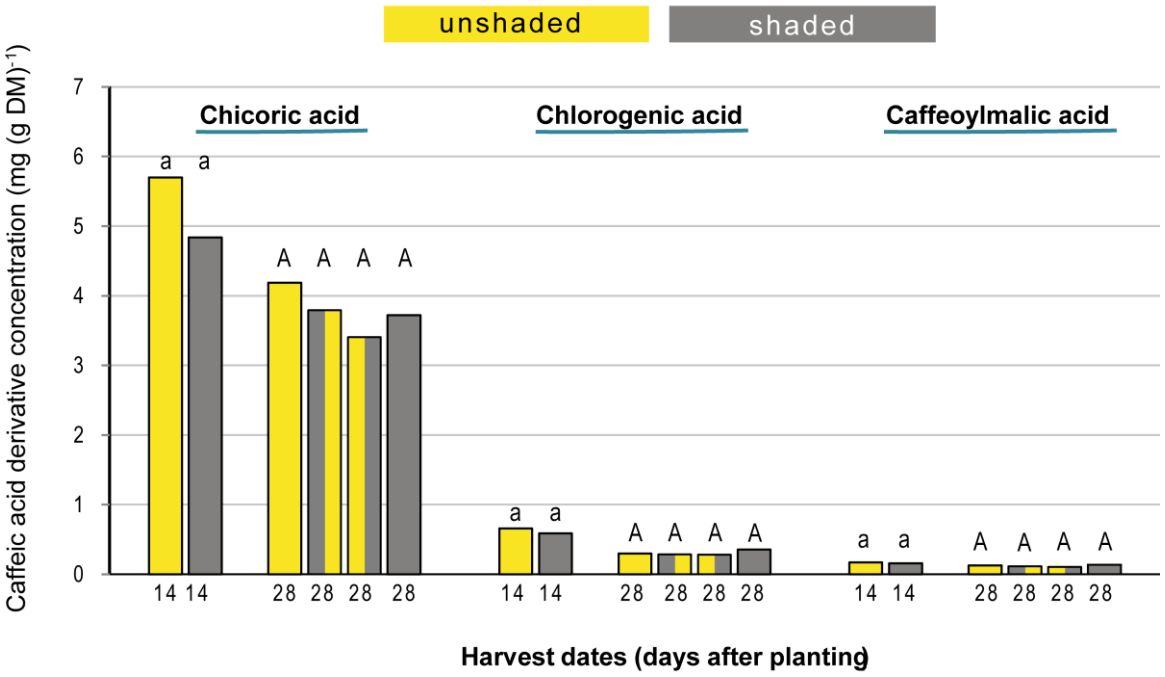

Figure 32: Concentration of chicoric acid (di-*O*-caffeoyltartaric acid), chlorogenic acid (5-*O*-caffeoylquinic acid) and caffeoylmalic acid related to dry matter (DM) of red Oak Leaf lettuce, cultivated for 14 and 28 days, respectively, at different light conditions. Yellow bars represent cultivation at 410 µmol m^{-2} s^{-1}, grey bars represent cultivation at 225 µmol m^{-2} s^{-1}. For each compound, iIdentical letters on top of bars show that these treatments do not differ significantly (mean, n = 3; Tukey's HSD test, α = 0.05)

These results show that, although closely related in their biosynthesis and ecological relevance (Crozier et al. 2007), caffeic acid derivatives and flavonoid glycosides have very differentiated regulation mechanisms. This has been suggested previously by Tattini et al. (2004) who detected a radiation induced change in the ratio of flavonoids to hydroxycinnamic acids in *Ligustrum vulgare* leaves.

After 14 days, plants had higher concentrations of flavonoid glycosides and caffeic acid derivatives than after 28 days (fig. 31 and fig. 32). However, it is not clear whether the biosynthesis of phenolics was higher in younger plants than in older ones or if this is just a “dilution effect”. According to Hohl et al. (2001), inner leaves of lettuce heads contain less flavonoids than outer leaves because they are not directly exposed to radiation. Hence, a dilution effect might occur in larger heads when the ratio of outer to inner leaves per head decreases: Even if the outer leaves synthesize flavonoids at the same rate like in younger plants, the overall concentration would be lower.

4.2.4 Summary and outlook for experiment 1

The results show that radiation conditions prior to harvest have a larger impact on the concentration of phenolic substances in marketable lettuce heads than earlier conditions, at least for the flavonol and flavone glycosides. While quercetin glycosides responded sensitively to radiation reduction from 410 to 225 µmol m^{-2} s^{-1}, this was not significant with the cyanidin glycoside or caffeic acid derivatives. This demonstrated a strong structure dependency.

None of the studied health promoting substances displayed permanently reduced concentrations due to temporarily reduced PPFD in early growth stages and, additionally, biomass accumulation in younger plants was less affected by reduced radiation than in older ones. These results suggest that it may well be possible to save considerable amounts of energy for heating in greenhouses by applying transparent daytime energy screens during the first weeks of lettuce cultivation in the cool season without experiencing significant losses in health promoting phenolic substances or biomass.

4.3 Influence of radiation – Experiment 2: Greenhouse

4.3.1 Influence of cultivar

Throughout ontogeny, red Oak Leaf lettuce had a higher number of leaves, diameter and head mass than red Lollo (tab. 2). The concentration of phenolic compounds in lettuce can be greatly influenced by genotype (Gazula et al. 2005) but red Lollo and red Oak Leaf lettuce showed only quantitative and no qualitative differences. Concentrations were mostly higher in red Lollo which is largely in line with reports by DuPont et al. (2000). Multiple regression analysis listed the cultivar as influential factor on quercetin-3-*O*-(6''-*O*-malonyl)-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide, and chicoric acid concentration (tab. 3).

4.3.2 Plant growth characteristics

Tab. 2 presents head mass, number of leaves, and head diameter per cultivar at the three harvest dates. As intended, the plants were in three different growth stages (see fig. 33). Twelve days after planting, head mass and number of leaves of both cultivars were still low. The leaves formed an open, flat rosette in which all of them were exposed to radiation. From 12 to 21 DAP, head mass increased only moderately while there was a remarkable increase in diameter. The rosette was much denser at the second harvest date and not as flat as before, with leaves partly overlapping. From 21 to 35 DAP, there was a marked increase in head mass, while the diameter did not increase as much as before. Independent of cultivar, there was a significantly higher increase in head mass from 21 to 35 DAP compared to the earlier period, while the increase in diameter was higher from 12 to 21 DAP compared to the later period.

The results show that plants were transitioning from expanding their surface to accumulating biomass around the second harvest date. This is supported by Krug et al. (2002) stating that field grown head lettuce starts head formation when 13 to 20 true leaves have been formed. These results furthermore corroborate the visual assessment that plants had not yet developed a head 12 DAP but that this process had begun at 21 DAP and continued until 35 DAP.

In the following, the three studied growth stages will be named accordingly: pre-heading stage (harvest 12 DAP), heading stage (harvest 21 DAP), and mature heads (harvest 35 DAP), respectively.

Table 2: Plant growth characteristics of two red leaf lettuce cultivars, grown at low mean daily PPFD (43 - 230 µmol m^{-2} s^{-1}) for 12, 21, and 35 days, respectively. Diameter is given in centimeter, head mass in gram fresh matter. DAP = days after planting. (mean ± SD, n = 3)

DAP	Cultivar	Number of leaves	Diameter (cm)	Head mass (g FM)
12	red Oak Leaf	10.3 ± 0.2	28.8 ± 2.8	9.2 ± 1.5
21	red Oak Leaf	18.6 ± 2.5	37.7 ± 3.7	52.6 ± 24.3
35	red Oak Leaf	30.7 ± 5.0	41.3 ± 2.1	199.7 ± 66.5
12	red Lollo	7.3 ± 0.5	16.6 ± 1.2	7.5 ± 2.1
21	red Lollo	11.5 ± 2.1	26.7 ± 0.3	33.8 ± 13.1
35	red Lollo	21.0 ± 5.1	32.9 ± 0.6	137.0 ± 65.5

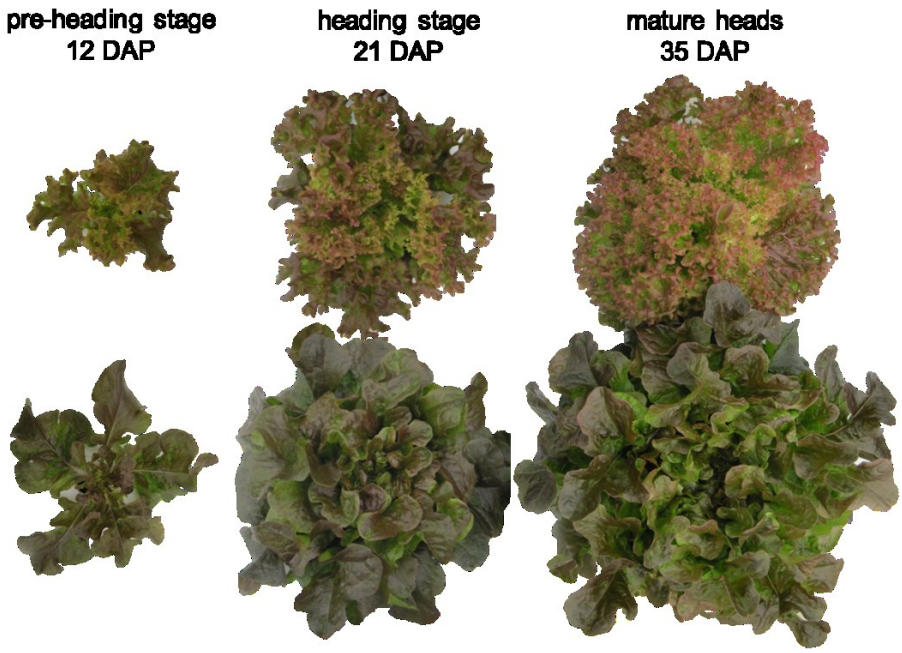

Figure 33: Red Lollo and red Oak Leaf lettuce (upper and lower row, respectively) at the three harvest dates. DAP = days after planting.

The light use efficiency between the first and second harvest was significantly lower than between the second and third harvest date: In the first phase, 0.4 g dry matter were gained per intercepted mol of photosynthetically active radiation whereas in the latter phase plants gained 0.8 g mol^{-1}. No significant differences between the cultivars were detected. The increase of light use efficiency during ontogeny is in line with the literature (Wheeler et al. 1993). It indicates that the photosynthetic capacity in the first interval was lower than in the second, as plants accumulated less biomass per intercepted mol of PAR.

4.3.3 Flavonoid glycosides

Results for flavonoid glycosides are presented in fig. 34 and tab. 3. Generally, all flavonoid glycosides were present in higher concentrations in plants in earlier growth stages than in later ones with the larger difference between pre-heading stage and heading stage than between heading stage and mature heads. Remarkably, the concentration of all flavonoid glycosides increased with PPFD even at the very low levels studied. The response depended on structure and growth stage.

In detail, regarding cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside, the lettuce plants' response to radiation varied between the growth stages considered. Multiple regression analysis revealed an interaction between PPFD and plant age: Increasing PPFD had a stronger influence on smaller plants than on larger ones. Furthermore, the increase with rising PPFD was becoming slower at higher PPFD levels (due to the negative influence of the square term of PPFD). Owing to the positive influence of the square term of DAP, the differences between growth stages were more pronounced between pre-heading and heading phase than between heading and mature heads.

Table 3: Results of multiple regression analysis: Equations presents the significantly influential factors on the single phenolic compounds of red leaf lettuce (n = 3, α = 0.01). DAP = days after planting, PPFD = photosynthetic photon flux density in µmol m^{-2} s^{-1}, cultivar = red Oak Leaf (1) and red Lollo (2). Cy3MG: cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside, Q3G: quercetin-3-*O*-glucoside, Q3MG: quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside, Q3Gc/ L7Gc: quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide. The value of R^2 displays the percentage of variance within the data that is explained by the equation (range = 0 - 1, 1 being 100%).

Compound	Equation	R^2
Cy3MG	=1.075774-0.148608*DAP+0.019459*PPFD-0.000028 *PPFD^2 +0.003040*DAP^2-0.000077*PPFD*DAP	0.88
Q3G	=0.061983-0.012242*DAP+0.033398*cultivar +0.001004*PPFD-0.000002*PPFD^2+0.000215*DAP^2	0.69
Q3MG	=6.472664-0.765445*DAP+2.181619*cultivar +0.020639*PPFD +0.013099*DAP^2	0.77
Q3Gc/ L7Gc	=0.761703-0.107507*DAP+0.236988*cultivar +0.007146*PPFD-0.000015*PPFD^2+0.001859*DAP^2	0.82
Chicoric acid	=1.003199-0.108660*DAP+0.893134*cultivar +0.002311*DAP^2-0.021650*cultivar*DAP	0.75
Chlorogenic acid	=0.932527-0.019782*DAP	0.45
Caffeoylmalic acid	=0.170214-0.001731*DAP	0.17

The interaction between PPFD and plant age is most interesting and has not been reported this clearly before. Regarding UV radiation, Behn et al. (2011) detected a similar age-dependent response when comparing leaves of different age. They cultivated red leaf lettuce for 21 days under UV B exclusion and subsequently transferred them to an environment with ambient UV B intensity for 2 days. Following this short term exposure to UV B radiation, cyanidin concentration only increased in inner (= young) leaves of lettuce heads. Because responsiveness generally decreased with increasing leaf age, they concluded that younger leaves have an increased requirement for cyanidin as photoprotectant because they have a lower capacity to utilize radiation energy compared to older ones. In the experiment presented here, the light use efficiency of young lettuce was indeed much lower than that of older plants: Per

intercepted mol of PAR, older plants gained twice the dry matter younger ones did. Concordantly, the PPFD required for maximum photosynthesis of lettuce plants increases with plant age (Sanchez et al. 1989). This implies an imbalance between captured light energy and its utilization in carbon fixation in early growth stages as has been suggested for juvenile tree leaves by Hughes et al. (2007). The surplus energy poses an oxidative threat to plant cells. This suggests that young plants were protecting themselves by anthocyanin accumulation and may explain the higher responsiveness of young plants detected in this experiment, even such low radiation intensities as studied.

Although the role of anthocyanins *in planta* is to some degree still subject to discussion, their protective role against photoinhibition and photooxidation in leaves is widely accepted (Hatier and Gould 2009): On the one hand, they absorb light in the green and yellow wavebands (500 - 600 nm) and, therefore, shield the photosynthetic apparatus against surplus radiation which could not be used for carbon assimilation (Gould 2004). On the other hand, they have a high antioxidant capacity and can counteract reactive oxygen species formed, for example, by photochemistry (Neill and Gould 2003). Especially during light-sensitive ontogenetic stages, e.g. in juvenile leaves whose photosynthetic apparatus is not yet fully functional, these qualities become obvious (Hughes et al. 2007). Young leaves are very valuable to plants and should, hence, be very effectively protected against harmful impacts (Reifenrath and Müller 2007).

The response of cyanidin glycoside to radiation in experiment 2 compared to experiment 1 is perplexing and appears to be inconsistent at first glance: Anthocyanins are known to be accumulated in high PPFD levels and UV radiation (Hatier and Gould 2009; Tsormpatsidis et al. 2010). When no significant influence was detected in experiment 1, it was assumed that either the PPFD level of 400 µmol m^{-2} s^{-1} was not high enough to trigger a response or the low-key response is due to the lack of UV radiation. While these explanations are plausible, they are challenged by the second experiment: Anthocyanins responded very sensitively to PPFD at even lower levels, likewise without UV B and only below 50% of ambient UV A.

What was happening here? As mainly UV B is biologically active, it is unlikely that the low intensity UV A was the crucial influence. A closer look at the

spectra incident in growth chamber and greenhouse (see appendix, p.122-124, fig. 48, 49, 50 and tab. 6 for details) revealed that the relative proportion of blue light (450 - 500 nm) is much higher in the greenhouse than in the growth chambers. According to Stutte (2009), blue light triggers anthocyanin biosynthesis in red leaf lettuce. Although there was a small fraction of blue light in the growth chambers it might just not have been enough to have strong impact on anthocyanin biosynthesis.

Alternatively, anthocyanin concentration may have approached a saturation level in experiment 1 and therefore only displayed very small differences between the treatments. Fig. 34 suggests such a saturation level for cyanidin glycoside in mature heads as PPFD increases. Ranging from 0.6 - 0.8 mg cyanidin glycoside per gram dry matter, the values obtained for mature red Oak Leaf lettuce heads in experiment 1 are correspond well to the values obtained for mature Oak Leaf heads and lie within the scope of an extrapolation of said graph. This explanation is very appealing, yet it is debatable if those plants really reached maximum anthocyanin biosynthesis. Lettuce plants were observed to obtain a darker coloration (than observed in experiment 1 and 2) when exposed to summer sunlight, putatively due to anthocyanin accumulation. Therefore, the “saturation hypothesis” is presented as one possible explanation amongst others. The summer sunlight presumably had a higher PPFD and relative percentage of UV radiation. It is therefore not possible to tell which (if any of these two) factors induced the darker coloration.

The greenhouse temperature may also have interfered. Although the mean temperature of 16 °C (min = 11.7 °C, max = 23.6 °C) was very close to the growth chamber conditions, it is unclear how low the temperature has to be to have an impact. Moreover, the temperature impact might be more complex, too, for instance interacting with changing PPFD. Overall, the climate was naturally more variable in the greenhouse which may additionally influence the regulatory processes.

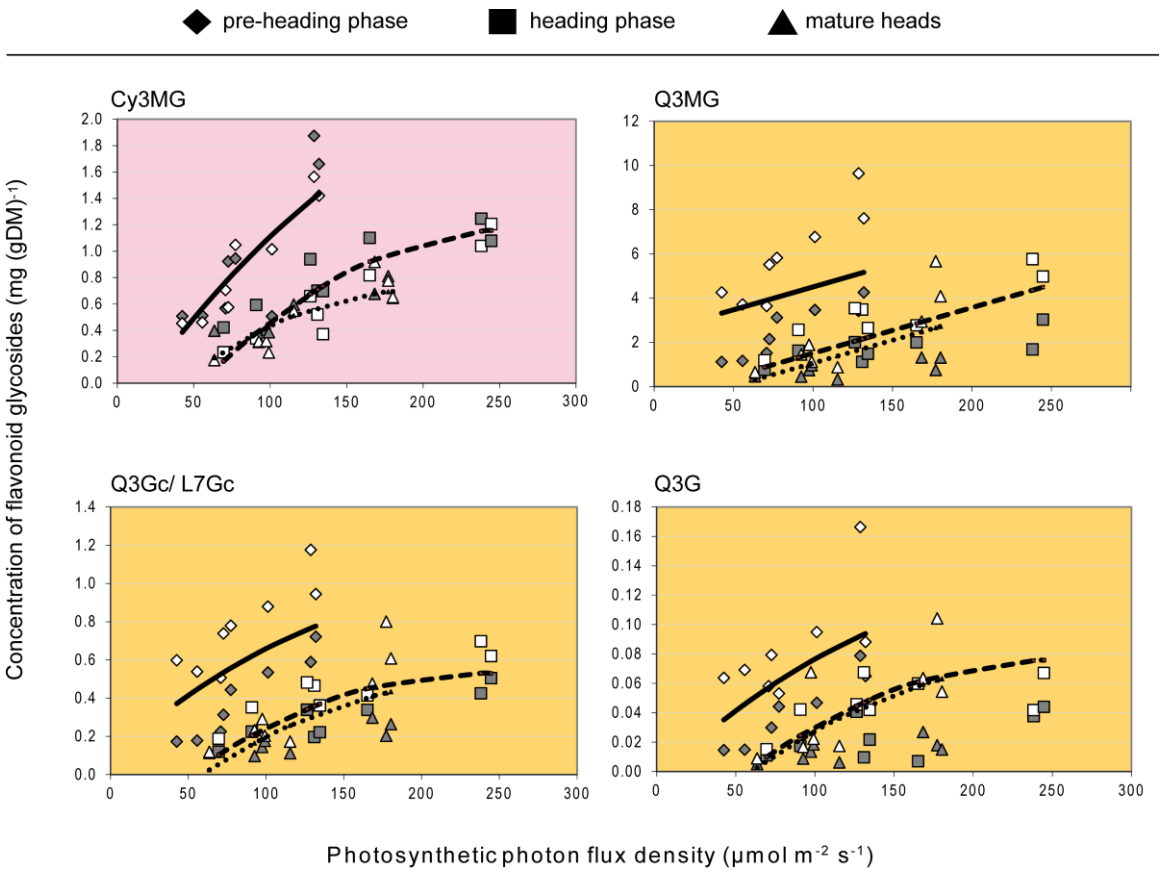

Figure 34: Concentration of flavonoid glycosides per gram dry matter (DM) of red leaf lettuce in three different growth stages in relation to photosynthetic photon flux density (in µmol m^{-2} s^{-1}). The relationship was evaluated via multiple regression analysis. Grey symbols represent values of red Oak Leaf lettuce, white symbols represent red Lollo values. Diamonds represent values of pre-heading phase, squares represent values of heading phase while triangles represent values of mature heads. The solid line is based on the values of pre-heading stage, the broken line is based on values of heading stage while the dotted line is based on values of mature heads (n = 3). Cy3MG: cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside, Q3G: quercetin-3-*O*-glucoside, Q3MG: quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside, Q3Gc/ L7Gc: quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide.

The concentration of quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside, quercetin-3-*O*-glucoside, and of quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide also increased with rising PPFD and decreased with plant age. Between pre-heading and heading plants the difference regarding concentration was very pronounced. Between heading plants and mature heads, on the other hand, there was hardly any detectable difference. The general increase of the quercetin glycoside concentration with radiation is in line with previous reports (Oh et al. 2009; Behn et al. 2011). Yet, it has not been demonstrated before for such low PPFD levels.

Other than regarding cyanidin glycosides, there was no interaction detected between PPFD and plant age regarding quercetin glycoside concentration. Accordingly, Behn et al. (2011) also found the UV B-response of cyanidin to be stronger influenced by leaf age than the response of quercetin whose concentration increased in all of the studied leaf ages.

Detected effects were clearly structure dependent: On the one hand they were related to the aglycone: Cyanidin glycosides responded plant age-dependent while quercetin glycosides did not. On the other hand, the glycosidic moiety appears to play a role, too: In the radiation levels studied, quercetin-3-*O*-(6''-*O*-malonyl)-glucoside concentration increased linearly with PPFD. Unlike this, quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide concentration were negatively influenced by the square term of PPFD, i.e. the increase was not linear and abated with rising PPFD.

All studied flavonoid glycosides displayed a remarkable, unexpectedly strong response to changes in PPFD level between approximately 50 – 150 µmol m^{-2} s^{-1} which was independent of plant age. This demonstrates an impressing sensitivity of the regulative mechanisms.

As mentioned earlier, one major aspect of progressing plant age in the vegetative growth phase of lettuce is the increasing number of leaves accompanied by a change in plant architecture: The formation of a more or less dense head changes the distribution of radiation within the plant. The relative decrease of light-exposed, flavonoid-synthesizing tissue per plant may explain the decrease of flavonoid glycosides with progressing head formation measured here.

Yet, according to Kubasek et al. (1998), flavonoid biosynthesis regulation is influenced by environmental as well as developmental factors. When studying the induction of four flavonoid biosynthesis genes in *Arabidopsis thaliana* seedlings, they found a higher induction potential in three day than in seven day old seedlings. Despite obvious differences between the plants studied by Kubasek et al. (1998) and in this experiment, those results may at least be partly transferable to lettuce: The interaction between plant age and PPFD regarding cyanidin glycoside allows for the speculation that the decreasing concentration with plant

age may not only be due to the afore mentioned dilution effect but also be influenced by growth-stage dependent variation in the induction capacity of genes involved in biosynthesis. It would therefore be interesting to take a closer look and compare the induction of genes at the junction leading to quercetin or cyanidin biosynthesis – for instance the mRNA levels of flavonol synthase and dihydroflavonol 4-reductase, respectively (Treutter 2010) – in younger compared to older plants. Yet, considering the high number of levels where regulatory processes can take place following transcription (stability of mRNA, translator effectivity, enzyme activation and more), it might be more interesting to study the quantity and activity of the respective enzymes.

In experiment 1, the question remained unanswered if the decrease of quercetin concentration with plant age was merely attributable to a dilution effect or if varying biosynthetic capacities were involved. As the regression analysis in experiment 2 suggests that PPFD has the same effect on quercetin glycoside concentration independent of plant age, the dilution effect is more probable.

4.3.4 Caffeic acid derivatives

The results for caffeic acid derivatives are presented in fig. 35 and tab. 3. Independent of growth stage, PPFD had no significant influence on the concentration of any of the caffeic acid derivatives. Unlike PPFD, progressing ontogeny did have an influence on the concentration of all three caffeic acid derivatives: They decreased with plant age, however to varying degrees.

The decrease of chicoric acid concentration with plant age was counteracted by the positive influence of the square term of DAP. Hence, it was markedly higher in the pre-heading than in the heading stage, while the latter did not differ as much from the mature heads' concentration. A significant influence of the cultivar on chicoric acid concentration was detected. Chlorogenic and caffeoylmalic acid were only significantly influenced by DAP, i.e. plant age.

The results that none of the three studied caffeic acid derivatives was influenced by PPFD is not in line with Oh et al. (2009) who found an increase of caffeic acid derivatives with increased radiation. However, they exposed small lettuce plants to 800 µmol m^{-2} s^{-1} for one day – this is much higher than the PPFD incident in this experiment (50 – 250 µmol m^{-2} s^{-1}). According to Fu et al. (2012),

lettuce suffers no stress under 100 – 400 µmol m^{-2} s^{-1} but serious stress under 800 µmol m^{-2} s^{-1}. Furthermore, greenhouse glass absorbs UV radiation to a large degree (see appendix, p. 123, fig. 49 for details) and phenolic acids are discussed as UV protectants in red leaf lettuce (García-Macías et al. 2007). Hence, incident radiation in this experiment may have lacked intensity and UV wavelengths in order to impact caffeic acid derivative biosynthesis.

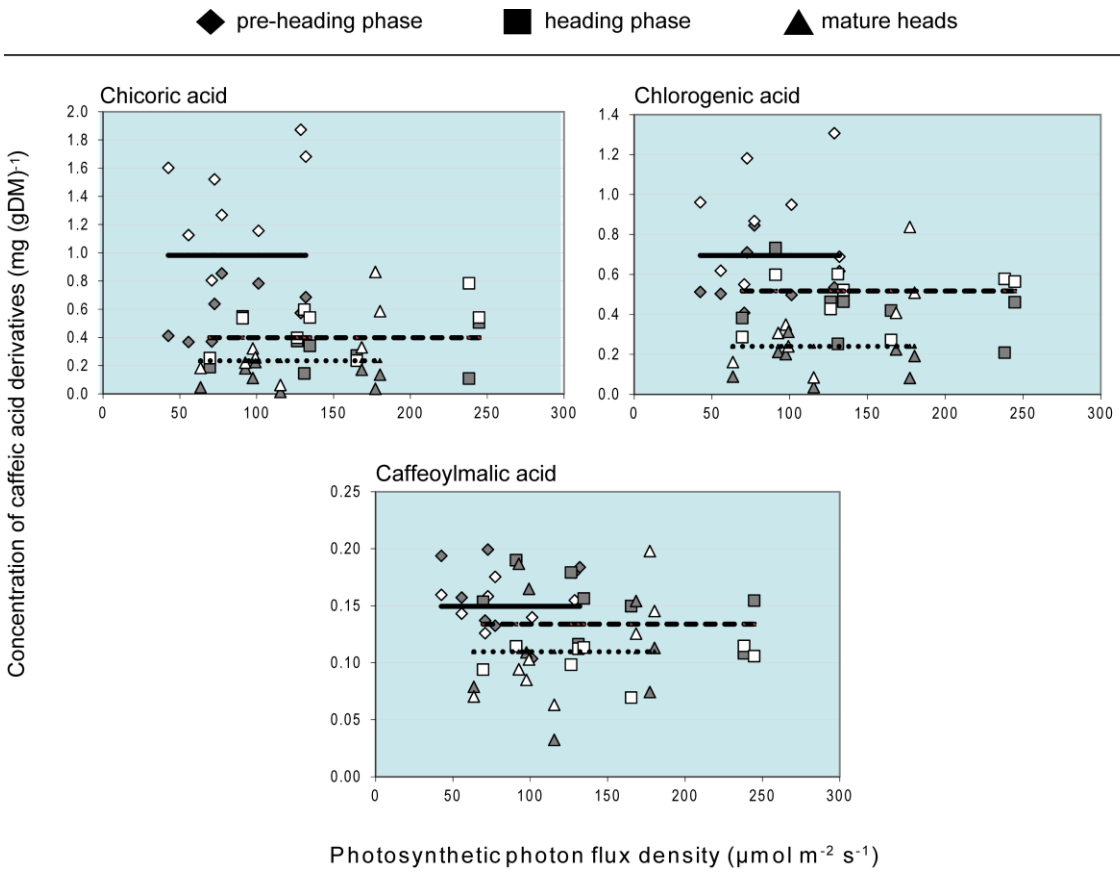

Figure 35: Concentration of caffeic acid derivatives per gram dry matter (DM) of red leaf lettuce in three different growth stages in relation to photosynthetic photon flux density (in µmol m^{-2} s^{-1}). The relationship was evaluated via multiple regression analysis. Grey symbols represent values of red Oak Leaf lettuce, white symbols represent red Lollo values. Diamonds represent values of pre-heading phase, squares represent values of heading phase while triangles represent values of mature heads. The solid line is based on the values of pre-heading stage, the broken line is based on values of heading stage while the dotted line is based on values of mature heads (n = 3).

4.3.5 Summary and outlook for experiment 2

The results show that flavonoid glycosides responded remarkably sensitive to changes in very low level PPFD (43 – 230 µmol m^{-2} s^{-1}) whereas caffeic acid derivatives did not. This structure-dependency was further highlighted by the interaction between plant age and PPFD level regarding cyanidin glycoside concentration.

These results emphasize the great importance of taking into account the plant's developmental stage when studying its response to radiation.

For lettuce cultivation in greenhouses and the application of transparent energy saving screens this implies that screen application during red leaf lettuce production is possible without reducing the concentration of chicoric, chlorogenic or caffeoylmalic acid, no matter in which growth stage plants are shaded. Unfortunately, this is not true for cyanidin and quercetin glycosides. Yet, in mature heads losses due to reduced radiation were smaller than with younger plants regarding 3 out of 4 flavonoid glycosides.

The high concentration of polyphenols in pre-heading leaf lettuce and their response to shading may be especially interesting for producers of baby leaf lettuce.

4.4 Influence of temperature – Experiment 3: Growth chamber

4.4.1 Influence of cultivar

The two cultivars of red leaf lettuce showed significant quantitative but no qualitative differences regarding most of the phenolic compounds and growth parameters (tab. 4). In detail, head mass and dry matter content were higher with red Oak Leaf than with red Lollo lettuce whereas the concentrations of cyanidin, quercetin and luteolin glycosides as well as of chicoric and chlorogenic acid were higher in red Lollo than in red Oak Leaf lettuce (data not shown). This is in line with previous studies (DuPont et al. 2000). No interactions were detected between temperature treatment and lettuce cultivar (tab. 4). Therefore, the average effect of the temperature treatments on both cultivars will be displayed in the following.

4.4.2 Plant growth characteristics

Plants harvested after 200 DD had a mean head mass of 42.8 ± 13.7 g FM and will further be referred to as “small heads” while plants harvested after 400 DD, with a mean head mass of 242.9 ± 35.5 g FM, will be referred to as “mature heads”.

Small heads that were cultivated cool for 26 days had a significantly higher mass than small heads cultivated warm for 13 days (fig. 36, tab. 4). Regarding mature heads, cool-cultivated plants also had a significantly higher head mass than warm-cultivated ones while head mass of plants that had been transferred between temperature regimes lay in between (fig. 36). Generally, lettuce heads were heavier the more days they were cultivated. This can be explained by the different total light integrals the plants experienced (see materials and methods, subsection 3.1.2). Small heads had a mean number of leaves of 18.1 ± 1.5, without significant differences between warm- and cool-cultivated ones (fig. 36, tab. 4). Mature heads had on average developed 39.4 ± 4.4 leaves per plant, with significant differences between plants from different treatments: Plants cultivated cool all the time or only for the first weeks had a significantly higher number of leaves than plants cultivated warm for the first weeks or all the time (fig. 36, tab. 4). Apparently, the temperature regime in earlier growth stages determined the number of leaves the mature heads developed.

Table 4: Influence of temperature and cultivar on growth characteristics and the concentration of phenolic compounds, assessed by two-way ANOVA (F-test; factor 1: treatment, factor 2: cultivar; n = 2). Data was evaluated separately for the two growth stages investigated. Cy3MG: cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside, Q3G: quercetin-3-*O*-glucoside, Q3MG: quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside, Q3Gc/ L7Gc: quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide. Given p-values display the probability that the observed differences occurred by chance.

		p-values for		
Characteristics	**Growth stage**	**Temperature**	**Cultivar**	**Interaction**
Plant growth				
Head mass	Small heads	0.001	0.002	0.13
	Mature heads	< 0.0001	< 0.0001	0.17
Number of leaves	Small heads	0.07	0.002	0.19
	Mature heads	0.002	< 0.0001	0.87
Dry matter content	Small heads	0.045	0.003	0.26
	Mature heads	0.009	0.003	0.52
Anthocyanidin glycoside				
Cy3MG	Small heads	0.04	0.06	0.19
	Mature heads	0.02	< 0.0001	0.23
Flavonol and flavone glycosides				
Q3G	Small heads	0.73	0.16	0.42
	Mature heads	0.84	0.02	0.92
Q3MG	Small heads	0.44	0.02	0.45
	Mature heads	0.79	0.003	0.92
Q3Gc/ L7Gc	Small heads	0.13	0.02	0.23
	Mature heads	0.81	0.003	0.86
Caffeic acid derivatives				
Chicoric acid	Small heads	0.84	0.006	0.75
	Mature heads	0.78	0.03	0.89
Chlorogenic acid	Small heads	0.11	0.03	0.94
	Mature heads	0.70	0.05	0.88
Caffeoylmalic acid	Small heads	0.004	0.89	0.46
	Mature heads	0.27	0.49	0.76

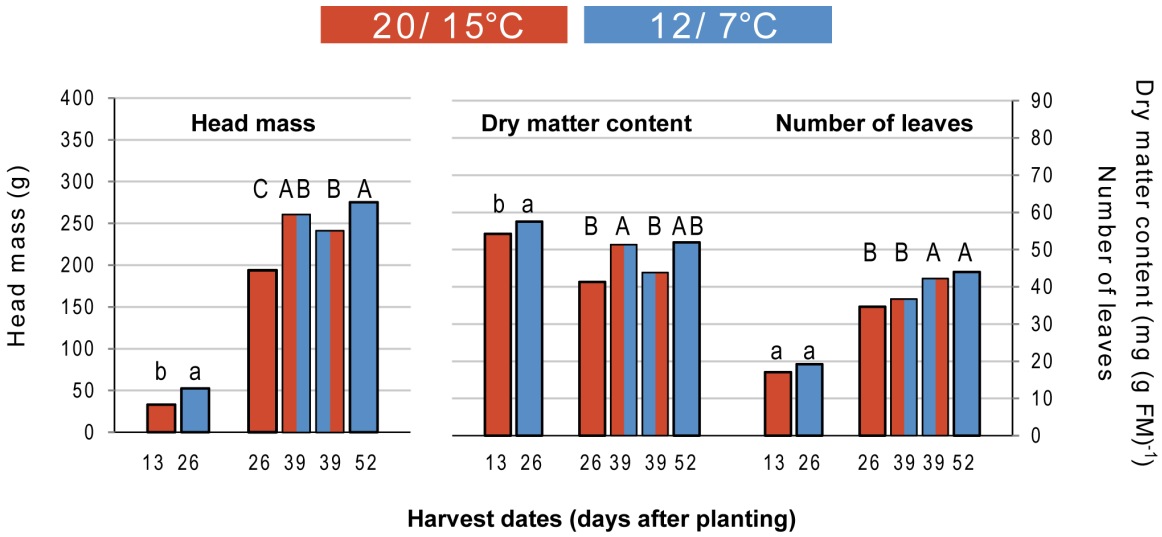

Figure 36: Head mass in gram fresh matter (FM), dry matter content (milligram per gram FM) and number of leaves of red leaf lettuce, cultivated in different temperature regimes for a different number of days. Blue bars represent cultivation at 12/ 7 °C, red bars represent cultivation at 20/ 15 °C. For detailed description of the treatments, please see caption of figure 9: "Harvest schedule", subsection 3.1.3. Identical letters on top of bars show that these treatments do not differ significantly (n = 2; Tukey's HSD test, α = 0.05).

Cool-cultivated small heads had higher dry matter content than warm cultivated ones (fig. 36, tab. 4). Cool-cultivated mature heads, as well as those that had been transferred from warm to cool, had a higher dry matter content than warm-cultivated ones while that of plants which had been transferred from cool to warm lay in between (fig. 36, tab. 4). In general, differences between small heads and mature heads were not as pronounced as regarding head mass (fig. 36), although small heads on average had higher dry matter content than mature heads (5.6% and 4.7%, respectively).

Previous studies (Boo et al. 2011) compared plants' phenolic content after having subjected them to different temperatures for the same number of days. Therefore, growth characteristics of plants cultivated cool and warm for 26 days were compared in this experiment, too (tab. 5). Plants that have been cultivated warm for 26 days had a much higher head mass (194.1 g FM) and number of leaves (34.7) than those cool-cultivated for 26 days (52.5 g FM, 19.2; fig. 36, tab. 5) indicating that they are in a more advanced growth stage than cool-cultivated ones. These differences are much more pronounced than between small heads or between mature heads (fig. 36, tab. 4, tab. 5). Additionally, after 26 days, dry matter content was higher in cool- than in warm-cultivated plants

(5.8%, 4.1%; fig. 36, tab. 5). Obviously, the differences regarding growth characteristics are generally bigger between plants cool- and warm-cultivated for 26 days than between plants harvested after approximately the same day-degrees. Thus, in order to single out the effect of temperature alone and to obtain results of practical relevance, it was considered more meaningful to compare plants in corresponding growth stages in this experiment.

Table 5: Influence of temperature and cultivar on growth characteristics and concentration of phenolic compounds after cultivation at 12/ 7 °C and 20/ 15 °C (day/ night) for 26 days, assessed by two-way ANOVA (F-test; factor 1: growth stage, factor 2: cultivar; n = 2). Cy3MG: cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside, Q3G: quercetin-3-*O*-glucoside, Q3MG: quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside, Q3Gc/ L7Gc: quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide. Given p-values display the probability that the observed differences occurred by chance.

	p-values for		
Characteristics	**Temperature**	**Cultivar**	**Interaction**
Plant growth			
Head mass	< 0.0001	0.002	0.01
Number of leaves	< 0.001	0.001	0.30
Dry matter content	0.01	0.04	0.65
Anthocyanidin glycoside			
Cy3MG	0.02	0.02	0.40
Flavonol and flavone glycosides			
Q3G	0.10	0.05	0.44
Q3MG	0.009	0.01	0.21
Q3Gc/ L7Gc	0.005	0.01	0.11
Caffeic acid derivatives			
Chicoric acid	0.01	0.03	0.50
Chlorogenic acid	0.11	0.11	0.83
Caffeoylmalic acid	0.01	0.67	0.95

4.4.3 Flavonoid glycosides

The concentration of cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside was significantly higher in cool-cultivated than in warm-cultivated small heads (fig. 37, tab. 4). Regarding mature heads, the first warm then cool-cultivated plants had the highest mean concentration of cyanidin glycosides – significantly higher than plants cultivated first cool then warm (fig. 37, tab. 4). In contrast, there was no significant difference between plants cultivated warm or cool all the time (fig. 37, tab. 4).

Boo et al. (2011) reported an elevated anthocyanin concentration in lettuce due to low temperature. In their experiment, lettuce was grown for the same number of days (six weeks) at temperatures as diverse as 30/ 25 °C and 13/ 10 °C. Plants from those treatments probably differed strongly regarding their growth stages (see subsection 4.4.2 for comparison). In the experiment presented here, comparing plants cultivated cool or warm for the same number of days produced significant differences, too (26 days; tab. 5, fig. 37). Yet, cool-cultivated small heads also contained higher concentrations than warm-cultivated small heads, i.e. plants in a corresponding growth stage (tab. 4, fig. 37). That is to say that this enhancing effect of low temperature was significant even when developmental bias was excluded.

Nevertheless, the enhanced anthocyanin concentration appeared to have been transient. While cool-cultivated small heads had a 59% higher concentration than warm-cultivated small heads, regarding mature heads, no significant difference was detected between warm- and cool-cultivated ones (fig. 37, tab. 4). The only treatments that induced significant differences among mature heads were the transfer treatments: Plants that had been transferred to the cool regime contained a 52% higher cyanidin glycoside concentration than plants transferred to the warm regime. While anthocyanins were accumulated upon sudden exposure to lower temperatures, their concentration vice versa apparently decreased when the stimulus was gone. Turnover rate of anthocyanins can be very high, especially in warm temperature: Only roughly 20% of the original concentration was left already 2 days after the stimulus ceased, when tested by Olsen et al. (2009) on *A. thaliana*.

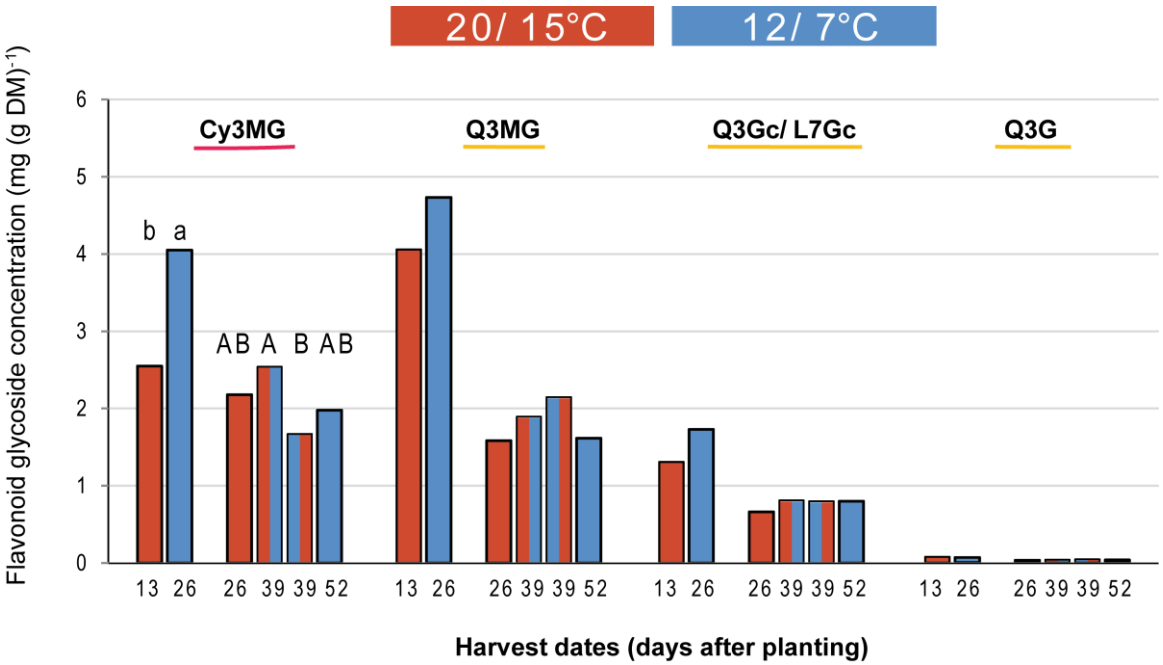

Figure 37: Concentration of flavonoid glycosides related to dry matter (DM) of red leaf lettuce, cultivated in different temperature regimes for a different number of days. Cy3MG: cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside, Q3G: quercetin-3-*O*-glucoside, Q3MG: quercetin-3-*O*-(6´´-*O*-malonyl)-glucoside, Q3Gc/ L7Gc: quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide. For each compound, the first two bars represent small lettuce heads (days 13 and 26), while the other four bars (days 26, 39, 39, and 52) represent mature heads. For detailed description of the treatments, please see caption of figure 9, subsection 3.1.3. Identical letters on top of bars show that these treatments do not differ significantly (n = 2; Tukey's HSD test, α = 0.05).

The results indicate that on the one hand, the low temperature regime was more stressful to plants in an early than in a later growth stage. Additionally, plants were apparently able to acclimate to the low temperatures applied in this experiment: After 52 days of low temperature cultivation, anthocyanin concentration was not enhanced. In contrast, anthocyanin concentration differed between plants exposed to altered temperatures only 13 and 26 days ago.

When temperature is low, light intercepted by plants and supplied to the electron transport chain of the photosynthetic apparatus in chloroplast thylakoid membranes may eventually become overexcessive. Due to $NADP^+$ limitation, the overreduction of the electron carriers can eventually lead to the formation of ROS (Havaux and Kloppstech 2001; Edreva 2005a). Neill and Gould (2003) suggest that cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside acts as both antioxidant and light attenuator in red Lollo lettuce: Accumulation of red tautomer cyanidin glycoside in

the epidermis can counteract ROS formation indirectly by alleviating the oxidative load in photosynthetically active cells by absorbing part of the surplus photons. Apart from providing photoabatement by accumulating in epidermal cell vacuoles, anthocyanins can act as antioxidants in the cytosol of photosynthetic active cells and directly scavenge ROS (Neill and Gould 2003).

Superoxide anion radicals ($O_2^{\cdot-}$) are the “energy outlet” of an overexcited electron transport chain in chloroplasts (Edreva 2005a). According to Gould and Lister (2006), a growing body of evidence indicates that anthocyanins contribute to ROS control in plants although they possess an extremely efficient enzyme to scavenge $O_2^{\cdot-}$: superoxide dismutase (SOD). They propose anthocyanins may be of greatest use to plants when other antioxidants are not fully functional – which may just be the case in low temperature because SOD is an enzyme and may, therefore, not be able to work to full capacity in low temperature. It has been suggested that even enzymatic repair processes are slowed down by low temperature (Bilger et al. 2007).

In concert with ascorbic acid, anthocyanins effectively scavenge artificially induced $O_2^{\cdot-}$ in *A. thaliana* (Nagata et al. 2003). Interestingly, the degree of contribution varied even among the tested ecotypes.

The colorless tautomer of cyanidin-3-*O*-(6´´-*O*-malonyl)-glucoside expected to occur in red leaf lettuce cytosol, is a very effective scavenger of $O_2^{\cdot-}$(Neill and Gould 2003). If not scavenged, $O_2^{\cdot-}$ is relatively fast metabolized to hydrogen peroxide (H_2O_2) which has a longer half-life than $O_2^{\cdot-}$(1 ms compared to 2 - 4 µs), is able to permeate membranes and, thus, diffuse freely between organelles and even from photosynthetically active mesophyll to epidermal cells (Agati et al. 2007; Gill and Tuteja 2010). H_2O_2 has been described as a short- and long distance signal earlier (Laloi et al. 2004; Foyer and Noctor 2005) and would therefore be a promising candidate to also link anthocyanin accumulation in non-photosynthetic epidermal cells to $O_2^{\cdot-}$ produced by overreduced chloroplast electron transport chains. This signaling function has also been suggested by Gould and Lister (2006).

The connection between ROS production by overreduced ETC and anthocyanin accumulation implies a higher oxidative load in cells of small heads

than in mature heads, in this experiment. The reason may be greater temperature-sensitivity of small heads compared to older ones. Young lettuce plants supposedly have a lower photosynthetic capacity than older ones (see experiment 2). An underdeveloped photosynthetic apparatus might be impaired at less low temperature than a fully developed one.

Mature cool-cultivated plants may have been able to acclimate to low temperature by down-scaling their light-harvesting antennae and altering the chlorophyll a/b ratio (Havaux and Kloppstech 2001). Thereby, the amount of energy captured and funneled into the ETC can be reduced and photoabatement by anthocyanin accumulation would not be necessary. In this context, it would be interesting to investigate the concentrations of chlorophyll a/ b and carotenoids like xanthophyll. Young leaves may display increased photosensitivity due to insufficient photochemical quenching because of yet low xanthophyll biosynthesis (Gould and Lister 2006). The acclimation hypothesis is supported by the observation that cyanidin glycoside concentration is much higher in small cool-cultivated heads than in mature cool-cultivated heads. One might argue that this is just the before mentioned dilution effect due to changed head architecture. Yet, comparing small and mature warm-cultivated heads does not reveal a comparable decline (fig. 37).

Regarding quercetin-3-*O*-(6''-*O*-malonyl)-glucoside, quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide, and quercetin-3-*O*-glucoside concentration, there were no significant differences between small heads that were cultivated either cool or warm (fig. 37, tab. 4). Furthermore, there were no significant differences concerning these compounds between mature heads cultivated in different temperature regimes (fig. 37, tab. 4).

When warm- and cool-cultivated plants were compared after the same number of days (26 days), significantly higher concentrations of quercetin-3-*O*-(6''-*O*-malonyl)-glucoside and quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide were detected in cool-cultivated ones (tab. 5, fig. 37). However, data from experiment 1 and 2 in this thesis shows higher concentrations of quercetin glycosides in early compared to later stage-lettuce. Subsection 4.4.2 demonstrated that warm- and cool-cultivated plants in this experiment were in different growth stages after 26 days of treatment. This leads to the conclusion

that the observed higher concentrations in the cool-cultivated plants were not directly due to temperature.

Løvdal et al. (2010) published concordant results on leaves of tomato plants (*Solanum lycopersicum*): Quercetin glycosides were accumulated in response to increasing light intensity and nitrogen depletion but not to lowered temperature alone. Macronutrient concentration in the nutrient solution of this experiment was closely monitored to ensure the supply is sufficient. Furthermore, the applied PPFD was constant (247 µmol m^{-2} s^{-1}).

The lowest temperature in this experiment (7 °C) was applied outside of the photoperiod and, therefore, did not coincide with radiation. Only with radiation present the photosystems can be overexcited and lead to ROS production (Wise 1995). This interacting and enhancing effect of low temperature and radiation has also been reported for *Arabidopsis thaliana* (Leyva et al. 1995; Havaux and Kloppstech 2001). Havaux and Kloppstech (2001) emphasized that the combination of chilling and elevated PPFD is especially likely to induce photoinhibition and photooxidation.

This may explain why results from this experiment differ from those of Oh et al. (2009). Apart from the different time span investigated (one day there compared to several weeks here), they subjected lettuce plants to 4 °C coinciding with radiation. Furthermore, they reduced the temperature by 16 K to 4 °C while here it was only reduced by 8 K to 7 °C. The larger magnitude of change and the application of a lower temperature during the photoperiod can exert more severe stress on plants and thus lead to an enhanced response.

The conditions applied in this experiment were more realistic regarding lettuce production in greenhouses than the drastic conditions applied in other studies.

In agreement with Løvdal et al. (2010), it is concluded that in this experiment the cyanidin glycoside truly responded to changes in temperature alone while quercetin and luteolin glycosides did not.

4.4.4 Caffeic acid derivatives

Cool-cultivated small heads contained higher concentration of caffeoylmalic acid than warm-cultivated ones (fig. 38, tab. 4). However, among mature heads, this difference is not detectable any more (fig. 38, tab. 4). This supports the hypothesis that the applied conditions were more stressful to small heads than to larger ones.

Neither in small nor in mature heads, significantly different concentrations of chicoric acid or chlorogenic acid were detected between the temperature treatments (fig. 38, tab. 4). Twenty-six days after planting, cool-cultivated plants contained higher concentrations of chicoric and caffeoylmalic acid than warm-cultivated ones, but this could not be detected for chlorogenic acid (tab. 5). As is elucidated in subsection 4.4.2, these plants were in very different growth stages and previous results (experiments 1 and 2) show that lettuce plants have higher concentrations of caffeic acid derivatives in early than in later growth stages. Hence, the elevated concentrations can probably not be interpreted as the plants' response to low temperatures but rather as developmental bias.

Of the three caffeic acid derivatives that were evaluated, only the concentration of caffeoylmalic acid differed between plants cultivated in different temperature regimes, and only regarding small heads. This heterogeneity is in agreement with the literature, indicating differences amongst phenolic acids regarding their response to environmental impacts (Oh et al. 2009) and amongst results obtained by different studies (Grace et al. 1998; Løvdal et al. 2010; Zidorn 2010).

Among the studied compounds, caffeoylmalic acid does not comprise the highest number of antioxidant structures per molecule (only one *ortho* 3',4'-dihydroxy moiety whereas chicoric acid comprises one in each of the two caffeic acid moieties). Thus, its accumulation in small heads probably has a function different from the commonly described antioxidant. Structure-wise there is no specific similarity between caffeoylmalic acid and cyanidin-3-*O*-(6''-*O*-malonyl)-glucoside which could have explained why these two phenolic compounds were present in higher concentration in cool- than in warm-cultivated small heads.

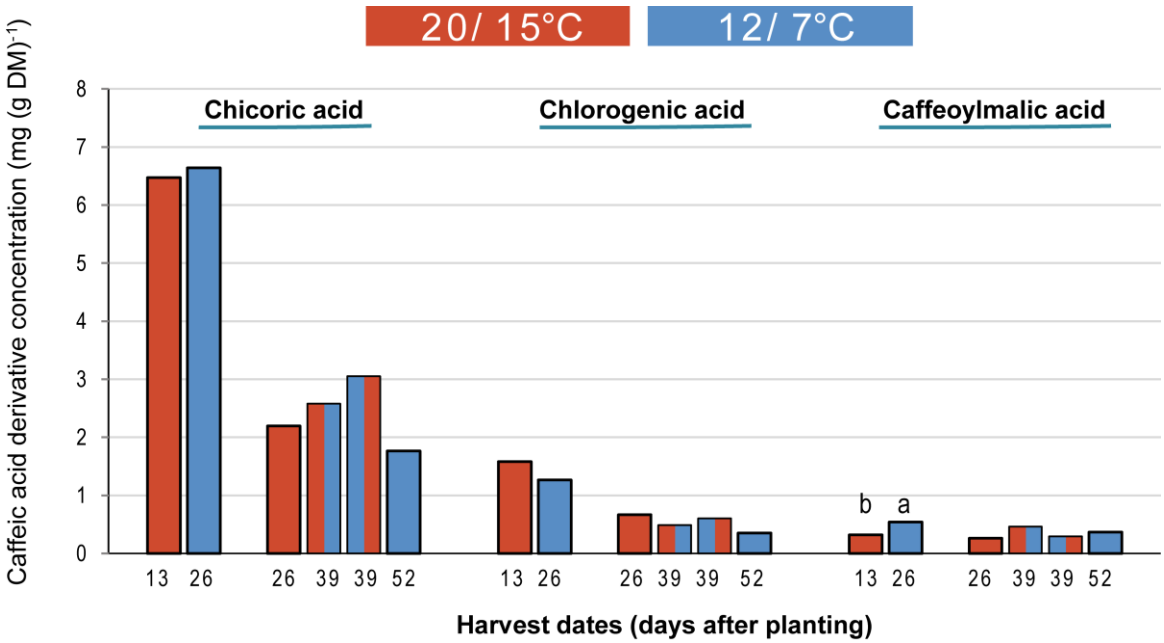

Figure 38: Concentration of caffeic acid derivatives related to dry matter (DM) of red leaf lettuce, cultivated in different temperature regimes for a different number of days. For each compound, the first two bars represent small lettuce heads (days 13 and 26), while the other four bars (days 26, 39, 39, and 52) represent mature heads. For detailed description of the treatments, please see caption of figure 9, subsection 3.1.3. Identical letters on top of bars show that these treatments do not differ significantly (n = 2; Tukey's HSD test, α = 0.05).

Unlike anthocyanins, caffeic acid derivatives do not absorb radiation in the wavelengths relevant for photosynthesis. Caffeic acid derivatives generally have their absorption maximum in the UV waveband and are therefore often considered UV protectants (García-Macías et al. 2007). However it is not very likely that UV played a role in this experiment as the applied radiation contained hardly UV radiation (HPS lamps; about 0.7% UV A and 0% UV B; see appendix, fig. 48, p.123, for more detail).

Løvdal et al. (2010) detected the strongest accumulation of caffeoyl derivatives in tomato leaves in response to a combination of high light, low nitrogen supply and low temperatures, indicating that temperature alone is not the trigger. Hence, the low-key impact detected in this experiment might be due to the constant PPFD, the close monitoring of nutrient solution, and application of the lowest temperature outside the photoperiod.

4.4.5 Summary and outlook for experiment 3

Low cultivation temperature affected flavonoid glycosides and caffeic acid derivatives in a strongly structure- and development-dependent manner. Anthocyanidin glycosides increased especially in small heads. The response in mature heads was detectable yet somewhat attenuated. Taken into account that small heads also accumulated caffeoylmalic acid, a greater temperature-sensitivity of younger compared to older plants is suggested.

Remarkably, neither of the quercetin glycosides responded to the reduction of the minimum temperature from 15 °C to 7 °C: Cool-cultivated plants did not contain higher concentrations of quercetin glycosides than those warm-cultivated as long as plants in corresponding growth stages were compared. Younger lettuce plants contain higher concentration of phenolic compounds and low temperature delays plant development. Results can be biased if this is disregarded.

The anticipation to obtain “climate friendly” greenhouse lettuce containing polyphenols in high concentrations has to be extenuated: When cultivated until large lettuce heads are formed, the concentration of phenolics will probably not be higher in cool- compared to warm-cultivated lettuce. However, especially in cool seasons, lettuce can be sold in earlier growth stages (100 - 150 g FM). These plants would not need as much time for cultivation and more plants could be grown per square meter (which are important economic aspects for producers). Furthermore, they are very likely to contain higher concentrations of phenolic compounds than larger heads. However, this has to be validated by greenhouse experiments under production conditions.

4.5 Structure-dependent responses – Summary and Conclusion

The three experiments that contributed to this thesis, revealed substantial differences in the response of major phenolic compounds in red leaf lettuce to certain abiotic factors. Two influential levels were detected: Caffeic acid derivatives differed greatly from flavonoid glycosides. But also among flavonoid glycosides there were remarkable differences, basically owing to the different aglycones. The glycosylation pattern (acylated or non-acylated glucosides or glucuronides) only played a minor role in contrast to cyanidin and quercetin which were of major influence. Quercetin glycosides responded very sensitively to changes in PPFD even when supplied on very low levels. Yet, they did not respond to the low temperature applied. The cyanidin glycoside did respond to low temperature and to very low PPFD, putatively due to its blue light fraction.

The complete lack of response from the caffeic acid derivatives to the applied PPFD levels and only one of them responding to low temperature, further emphasized the structure-dependency. Like flavonoids, these compounds also possess at least one *ortho* 3',4'-dihydroxy moiety – chicoric acid even comprises two. Yet, looking at these responses and those of quercetin and cyanidin glycosides, the antioxidant activity provided by this functional group does not appear pivotal in red leaf lettuce concerning the tested conditions.

Flavonoid biosynthesis is up-regulated by ROS (Guidi et al. 2008). According to Edreva (2005a), different components of the photosynthetic apparatus produce disparate types of ROS when over-excited (fig. 39) and Tattini et al. (2004) proposed that in response to different stressors those flavonoids would be synthesized which most effectively scavenge the respective ROS type produced.

Singlet oxygen is continuously produced during photosynthesis by energy transfer to molecular oxygen, mainly from triplet-state chlorophyll in photosystem II (Apel and Hirt 2004). The life time of triplet chlorophyll increases in excess radiation (Havaux and Kloppstech 2001). Yet, even under low-light conditions, singlet oxygen is a permanent by-product of photosynthesis (Buchert and Forreiter 2010).

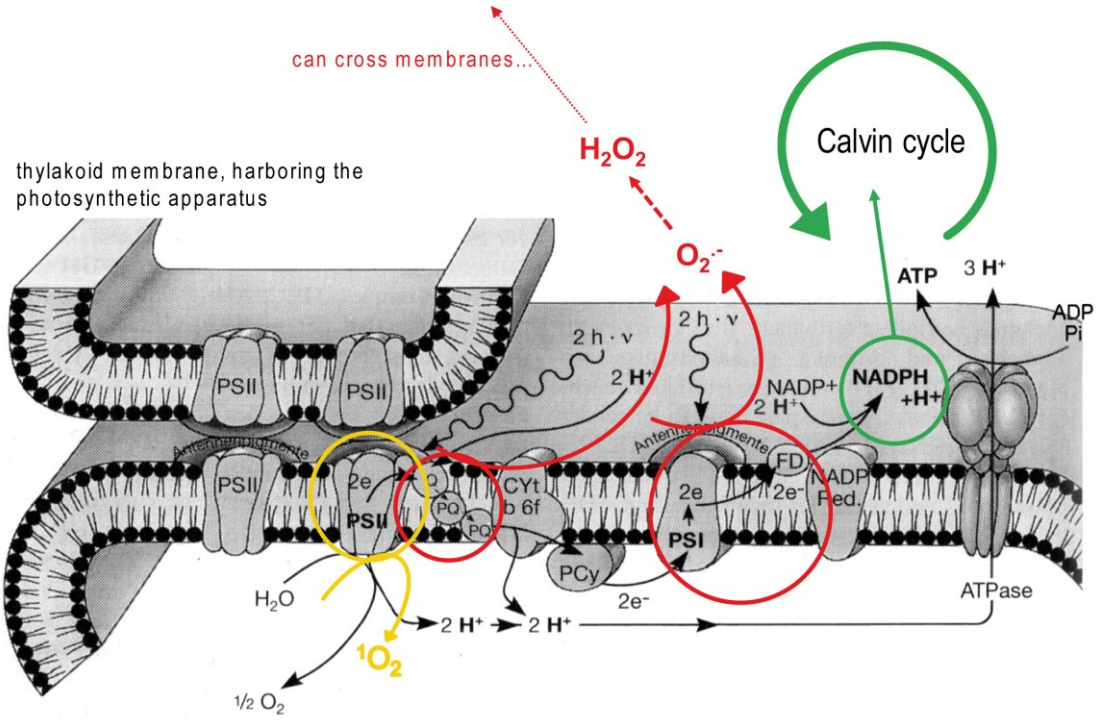

Figure 39: Electron transfer and ROS production during light-dependent parts of photosynthetic processes located in the chloroplast thylakoid membranes, depicted in a simplyfied scheme. When chlorophyll in the photosystems absorbs photons, electrons are sent on their passage through the electron transport chain until they reach the NADP reductase. This enzyme reduces $NADP^+$ to $NADPH+H^+$ which then transfers the energy so captured from the light-dependent part of photosynthesis to the light-independent, enzymatic part: the Calvin cycle. There are several options where the captured light energy can be diverted instead of reaching the $NADP^+$ reductase. Site of $^1O_2$ production by energy transfer is marked yellow, sites of $O_2^{\cdot-}$ production by electron transfer are marked red. PS I = photosystem I, PS II = photosystem II, Q, PQ = plastoquinone pool; FD = ferredoxin. Based on Lüttge et al. (2003), modified after Pfannschmidt (2003), Edreva (2005a), and Gould and Lister (2006)

Due to the hydroxyl group at C3 position which is activating the C ring double bond, quercetin is very effective against $^1O_2$ – the proposed mechanism being a cyclo-addition to the double bond leading to the formation of a 1,2-dioxetane intermediate (Tournaire et al. 1993). Neither cyanidin (fig. 40) nor the caffeic acid derivatives studied here possess that same structural feature.

Unlike $^1O_2$, superoxide anion radicals are formed by electron transfer. They are the energy outlet of the photosynthetic electron transport chain: Up to 25% of the electron flux can be diverted to molecular oxygen as alternative electron acceptor (Neill and Gould 2003). According to Edreva (2005a), leakage of electrons occurs mainly at electron carriers displaying the common feature of

easy electron exchange, like transition metals (iron in ferredoxin), or the availability of quinone structures as in plastoquinones.

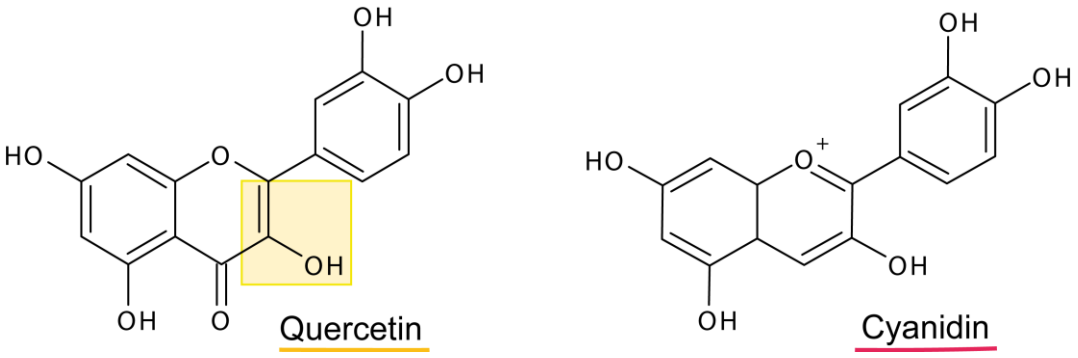

Figure 40: Cyanidin does not possess the structural feature (the hydroxyl group activating the double bond, highlighted in yellow) which according to Tournaire et al. (1993) makes quercetin a very effective scavenger of singlet oxygen.

Yet, also photosystem I itself can reduce O_2, likewise yielding $O_2^{\cdot -}$ via Mehler reaction[2]. Electron leakage occurs especially when $NADP^+$ cannot act as primary electron acceptor due to limited supply – this situation is established by suboptimal temperature via decelerating Calvin cycle activity which uses the energy provided by $NADPH+H^+$ and regenerates $NADP^+$ (Pfannschmidt 2003). Hence, the main ROS produced by low temperature alone (i.e. without strong light or other stressors) may be superoxide anion. Cyanidin-3-O-(6´´-*O*-malonyl)-glucoside is a very effective scavenger of $O_2^{\cdot -}$ (Neill and Gould 2003). Interestingly, cyanidin displays higher $O_2^{\cdot -}$ scavenging activity than quercetin which also applies to their respective 3-*O*-glycosides (Chun et al. 2003).

Based on the results obtained in this thesis, the hypothesis is put forward that the observed differential accumulation of quercetin and cyanidin glycosides is based on their disparate effectiveness against different ROS types which are formed in response to different elicitors. This would of course require differential regulation mechanisms. It has been reported than that $^1O_2$ is involved in the activation of early stress response genes which are different from those activated by $O_2^{\cdot -}$and H_2O_2 (Gill and Tuteja 2010). It would be very interesting in this context to monitor the formation of different ROS *in planta* in response to varying influential factors, also to different wavebands of visible radiation.

[2] Reduction of molecular oxygen in chloroplasts via Mehler reaction: $2e^- + O_2 \rightarrow 2O_2^{\cdot -}$ (Pfannschmidt 2003)

Their location *in planta* furthermore supports the hypothesis of discriminative roles: While quercetin has also been detected in the chloroplast where it is well located to scavenge short-lived $^1O_2$ molecules produced by photosynthetic processes (Agati et al. 2007), this is not the case for cyanidin which mainly occurs in vacuoles (Hatier and Gould 2009). Vacuolar cyanidin on the other hand may scavenge longer-lived H_2O_2 molecules diffusing from the chloroplast as suggested previously by Gould and Lister (2006). Spontaneously or catalyzed by SOD, $O_2^{\cdot -}$ dismutates to H_2O_2 (Edreva 2005a).

The quercetin-related part of this hypothesis is in line with a recently published review article, where the authors speculate that quercetin derivatives protect chloroplasts from $^1O_2$ induced by visible light (Agati et al. 2012). Anthocyanin accumulation in response to $O_2^{\cdot -}$ formation has also been proposed by Nagata et al. (2003), based on artificial $O_2^{\cdot -}$ creation in *A. thaliana*.. Several *ex vivo* experiments endorse the scavenging function of anthocyanins towards superoxide anions and hydrogen peroxide, as reviewed by Agati and Tattini (2010). Very recently Agati et al. (2013) proposed that dihydroxy flavonoids – including cyanidin – supported by ascorbic acid form a “secondary antioxidant system” in the vacuole which is otherwise poorly equipped with antioxidant machinery.

Additionally, op den Camp et al. (2003) proposed that dependent on PPFD level and duration of illumination, differential production of $^1O_2$ and $O_2^{\cdot -}$ was elicited, followed by differential gene induction in *Arabidopsis thaliana*. Mildest conditions triggered only $^1O_2$, but when radiation conditions grew harsher, singlet oxygen was accompanied by $O_2^{\cdot -}$. This corresponds anthocyanins being accumulated in response to high PPFD (Hatier and Gould 2009).

The hypothesis that quercetin accumulation is a reaction to $^1O_2$ and cyanidin accumulation to $O_2^{\cdot -}$, mediated by differential production of these ROS due to different abiotic stresses corresponds well to the results from the growth chamber experiments 1 and 3: Changing PPFD resulted in quercetin response but the same radiation spectrum at lower temperature prompted a response by cyanidin.

Yet, results from the greenhouse experiment (exp. 2), suggest that it might fall short of describing the whole picture which appears to be more complex: PPFD levels were so low that, according to the presented hypothesis, no cyanidin response should be detectable. However, the response was very clear. As suggested earlier, this may be related to the different light spectra: The higher relative proportion of blue light in the greenhouse compared to the growth chamber probably elicited the anthocyanin response.

Blue light may take effect by interacting with the photosynthetic apparatus: Photosystem II uses mainly blue wavelengths while photosystem I uses blue, red, and far red equally (Allen and Forsberg 2001). Hence, one might speculate that the higher intensity of blue light in the greenhouse may have led to higher frequency electron transfer also in the quinone-based ETC-part connecting photosystem I and II (see fig. 39), thus increasing the risk of $O_2^{\cdot -}$ production and in turn prompting anthocyanin accumulation. Alternatively, blue light could trigger anthocyanin biosynthesis simply by interacting with cryptochrome a receptor for blue light (Li and Yang 2007).

This thesis and the carefully conducted experiments therein offer valuable data as they made the responses of major phenolic compounds in red leaf lettuce to non-extreme abiotic factors visible in great detail by studying plants in controlled environments.

5 Conclusions

5.1 Influence of radiation, temperature and growth stage on flavonoids and caffeic acid derivatives in red leaf lettuce

Radiation conditions prior to harvest have a larger impact on the concentration of phenolic substances in marketable lettuce heads than the conditions in earlier weeks – at least for the flavonol and flavone glycosides. While quercetin glycosides responded sensitively to radiation reduction from 410 to 225 µmol m^{-2} s^{-1}, this was not significant with the cyanidin glycoside or caffeic acid derivatives. This demonstrates a strong structure dependency.

Flavonoid glycosides even respond remarkably sensitive to changes in very low PPFD ranges (43 – 230 µmol m^{-2} s^{-1}) whereas caffeic acid derivatives do not. The structure-dependency was further highlighted by the interaction between plant age and PPFD level regarding cyanidin glycoside concentration. Only concerning ontogeny, flavonoid glycosides and caffeic acid derivatives showed a uniform pattern: Concentrations decreased with plant age. These results emphasize the great importance of taking into account a plant's developmental stage when studying its response to radiation and to temperature.

Low cultivation temperature influenced flavonoid glycosides and caffeic acid derivatives in a strongly structure- and development dependent manner. In the very controlled environment studied here, anthocyanidin glycoside increased, especially in small heads. The response in mature heads was detectable yet somewhat attenuated. Taken into account that small heads also accumulated caffeoylmalic acid, a greater temperature-sensitivity of younger compared to older plants is suggested, possibly due to underdeveloped photosynthetic apparatuses. Quercetin glycosides did not respond to the low temperature. Previously reported contrasting effects may be biased by developmental effects as low temperatures delay plant development.

In conclusion, it appears that quercetin glycosides are very responsive to PPFD while cyanidin glycoside responds to PPFD as long as there is a sufficient fraction of blue light. Furthermore, cyanidin glycoside responds to low temperatures. While the concentration of flavonoid glycosides was generally higher in younger than in older plants, regarding cyanidin glycoside there

additionally was a more intense response to increasing PPFD in younger plants. Caffeic acid derivatives on the other hand appear not at all inducible by low to medium PPFD levels and only caffeoylmalic acid appears slightly responsive to low temperature.

Based on the experimental data and information taken from the literature, the conjecture is offered that quercetin glycosides may be accumulated in response to singlet oxygen produced by PAR-excited chlorophyll while cyanidin glycosides may be accumulated in response to superoxide anions produced by overreduced electron carriers of the photosynthetic electron transport chain (quinones and ferredoxin). The latter situation may however be created by low temperature, blue light or other scenarios not tested here (e.g., UV radiation).

It would be interesting to further investigate the production of different ROS types in the scenarios in detail and to analyze the activity of the studied phenolics to scavenge the respective ROS.

Another line of research would be to study the molecular biology to elucidate if young lettuce plants have a higher potential to synthesize anthocyanins than older ones. It would be interesting to investigate if the observed differences concerning responsiveness are also visible in quantity and activity of biosynthetic key enzymes.

5.2 Indications of the experimental results for lettuce cultivation in low energy greenhouses

None of the studied health promoting substances displayed permanently reduced concentrations due to temporarily reduced PPFD (from 410 to 225 µmol m^{-2} s^{-1}) and, additionally, biomass accumulation in younger plants was less affected by reduced radiation than in older ones. These results suggest may well be possible to save considerable amounts of energy for heating in greenhouses by applying transparent daytime energy saving screens during the first weeks of lettuce cultivation in the cool season without experiencing significant losses in health promoting phenolic substances or yield. However, these results have to be confirmed in the greenhouse under practical conditions before being recommended to producers.

At PPFD levels relevant for greenhouse lettuce production in cool seasons in Central Europe (43 to 230 µmol m^{-2} s^{-1}), screen application during red leaf lettuce production is possible without reducing the concentration of chicoric, chlorogenic or caffeoylmalic acid, no matter in which growth stage plants are shaded by the screen. Unfortunately, this is not true for cyanidin and quercetin glycosides although it remains to be elucidated if in such low PPFD, plants would also be able to compensate these losses when shading were only conducted in the first weeks of cultivation.

The generally high concentration of polyphenols in pre-heading leaf lettuce and their response to shading may be especially interesting for producers of baby leaf lettuce.

The anticipation to obtain “climate friendly” greenhouse lettuce containing high concentrations of polyphenols by low temperature cultivation has to be extenuated: When cultivated until mature lettuce heads are formed, the concentration of phenolics will probably not be higher in cool- compared to warm-cultivated lettuce. However, especially in cool seasons, lettuce can be sold in earlier growth stages (100 - 150 g FM). These plants would not need as much time for cultivation and more plants could be grown per square meter (which are important economic aspects for producers). They are, furthermore, very likely to contain higher concentrations of phenolic compounds than large heads. However, this has to be validated by greenhouse experiments under production conditions.

A combination of the discussed approaches to operate greenhouses with lower energy consumption seems promising in fall, winter, and early spring. Applying transparent energy saving screens only at the beginning of the cultivation period provides a well-insulated greenhouse and the shortfall in flavonoid glycosides and head mass can probably be compensated if screens are not applied anymore two weeks before harvest. If this leads to lower temperatures plant development may slow down but cyanidin glycosides should increase. This scenario would amalgamate the positive effects of the studied approaches.

6 Literature

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8 Eidesstattliche Versicherung

Name: Christine Becker

Matrikel-Nr.: 327484

Hiermit versichere ich, Christine Becker, an Eides statt, dass ich die vorliegende Dissertation mit dem Titel „Impact of radiation, temperature and growth stage on the concentration of flavonoid glycosides and caffeic acid derivatives in red leaf lettuce“ selbständig und ohne fremde Hilfe verfasst und keine anderen als die angegebenen Hilfsmittel benutzt habe. Die Stellen der Arbeit, die dem Wortlaut oder dem Sinn nach anderen Werken entnommen wurden, sind in jedem Fall unter Angabe der Quelle kenntlich gemacht.
Die Arbeit ist teilweise in wissenschaftlichen Fachzeitschriften veröffentlicht und die Daten sind in der Publikationsliste aufgeführt. Die Arbeit ist noch nicht (auch nicht an anderer Stelle oder in anderer Form) als Prüfungsleistung vorgelegt worden.

Ort, Datum Unterschrift

9 Lebenslauf

Christine Becker, geboren in Kassel

Seit März 2010:	Doktorandin an der Technischen Universität Berlin, wissenschaftliche Mitarbeiterin im Leibniz-Institut für Gemüse- und Zierpflanzenbau e.V., Großbeeren
	Promotionsthema: „Einfluss ökophysiologischer Faktoren auf Flavonoid- und Phenolsäuregehalt von Salat“
2003 – 2009	Studium der Biologie, Julius Maximilian Universität Würzburg
	Diplomarbeit: “Mechanismen eines Mutualismus: Einfluss von Samenqualität auf myrmekochore Systeme”
2002	Abitur, Scharnhorstgymnasium Hildesheim

10 Publications and conference contributions

Publications:

Experiment 2 is published in:

Title: “Unlike Quercetin Glycosides, Cyanidin Glycoside in Red Leaf Lettuce Responds More Sensitively to Increasing Low Radiation Intensity before than after Head Formation Has Started.”

Becker, C.; Klaering, M. Schreiner, H.-P. Kroh, L.W.; Krumbein, A. 2014.

Journal for Agricultural and Food Chemistry, DOI: 10.1021/jf404782n

Experiment 3 is published in:

“Cool-cultivated red leaf lettuce accumulates cyanidin-3-*O*-(6‘‘-*O*-malonyl)-glucoside and caffeoylmalic acid.”
Becker, C.; Klaering, H.-P. Kroh, L.W.; Krumbein, A. 2014.
Food Chemistry 146: 404-411.
http://dx.doi.org/10.1016/j.foodchem.2013.09.061

Experiment 1 is published in:

“Temporary reduction of radiation does not permanently reduce flavonoid glycosides and phenolic acids in red lettuce.”
Becker, C.; Klaering, H.-P. Kroh, L.W.; Krumbein, A. 2013.
Plant Physiology and Biochemistry 72: 154-160.
http://dx.doi.org/10.1016/j.plaphy.2013.05.006

“Diaspore trait preferences of dispersing ants.”
Reifenrath, K.; Becker, C.; Poethke, H.J. 2012.
Journal of Chemical Ecology, 38, p.1093-1104.
DOI 10.1007/s10886-012-0174-y

Talks:

“Response of flavonoid glycoside concentration in red leaf lettuce to PPFD is structure and partly growth stage dependent.”
Becker, C.; Kläring, H.-P.; Kroh, L.W.; Krumbein, A. 2013
7th International Workshop on Anthocyanins, Porto, Portugal; September 9-11th

“Does low temperature increase the concentration of flavonoid glycosides and phenolic acids in red leaf lettuce?”
Becker, C.; Krumbein, A.; Kroh, L.W.; Kläring, H.-P. 2013
Institutskolloquium IGZ, 23.04.2013, Großbeeren

„Neues aus Großbeeren: Einfluss niedriger Temperatur auf Salat“
Becker, C.; Kläring, H.-P.; Kroh, L.W.; Krumbein, A. 2013
ZINEG-Projekttreffen, 17.-18.4.2013, Hochschule Osnabrück

“Flavonoids and phenolic acids in lettuce – A Cooperation between IGZ and IACKR”
Christine Becker, Branimir Urlić, Maja Jukić Špika. 2013
Institute for Adriatic Crops and Karst Reclamation, 25.3.2013, Split, Croatia.

„Einfluss reduzierter Strahlung auf Wachstum und Konzentration phenolischer Substanzen in Salat“
Becker, C.; Kläring, H.-P.; Kroh, L.W.; Krumbein, A. 2012
ZINEG-Projekttreffen, 27.-28.3.2012, Leibniz Universität Hannover

„How to analyze anthocyanins in lettuce?”
Becker, C. 2012
Institutskolloquium IGZ, 20.03.2012, Großbeeren

“Influence of ecophysiological factors on flavonoid concentration in lettuce: Effects of reduced irradiation on selected aspects of lettuce metabolism.”
Becker, C.; Krumbein, A.; Kroh, L.W.; Kläring, H.-P. 2011
Institutskolloquium IGZ, 14.06.2011, Großbeeren

“ZINEG - The low energy greenhouse: Impact of reduced irradiation on growth and flavonoid synthesis of lettuce.”
Becker, C.; Krumbein, A.; Kroh, L.W.; Kläring, H.-P. 2011
GreenSys2011, Halkidiki, Greece, 05.06-10.06.2011.

„Stand der Salat-Experimente“
Becker, C.; Kläring, H.-P.; Kroh, L.W.; Krumbein, A. 2011
ZINEG-Projekttreffen, 29.-30.3.2011, Humboldt Universität Berlin

Posters:

„Nutzung von Tages-Energieschirmen in Gewächshäusern ohne Ertrags- oder Qualitätsverluste möglich?
Becker, C.; Kläring, H.-P.; Kroh, L.W.; Krumbein, A. 2012
Hans Eisenmann-Zentrum: Zentralinstitut für Agrarwissenschaften der Technischen Universität München. 3. Agrarwissenschaftliches Symposium, 20.09.2012. Tagungsband, 40-41.

“Impact of temporarily reduced irradiation on anthocyanin content of lettuce.”
Becker, C.; Kläring, H.-P.; Kroh, L.W.; Krumbein, A. 2012.
7th International Symposium on Light in Horticultural Systems. Wageningen, The Netherlands 14-18 October, Book of abstracts, 197.

“Impact of temporarily reduced irradiation on quercetin glycoside and phenolic acid concentration in lettuce.”
Becker, C.; Kläring, H.-P.; Kroh, L.W.; Krumbein, A. 2012
XXVIth International Conference on Polyphenols: Polyphenols Communications 2012, Vol. II, 23rd-26th July, Florence, Italy. ISBN 978-88-90711-0-3.

“Temporary shading of young red lettuce does not lead to permanent deficits in growth or concentration of health promoting phenolic compounds.”
Becker, C.; Kläring, H.-P.; Kroh, L.W.; Krumbein, A. 2012.
8th Plant Science Student Conference, IPK Gatersleben, Germany.04-06 June, Book of abstracts, 55.

„Qualitative und quantitative Unterschiede des Flavonidgehalts verschiedener Salatsorten und Endivie.“
Becker, C.; Kläring, H.P.; Kroh, L.W.; Krumbein, A. 2011.
Gartenbauwissenschaftliche Jahrestagung: Produkt- und Prozessinnovationen im Gartenbau, Leibniz Universität Hannover, 23.02.-26.02.2011. BHGL-Schriftenreihe 28, 95.Abstract, 47.

11 Appendix

11.1 List of abbreviations

ACN – acetonitrile
ANS – anthocyanidin synthase
CHS – chalcone synthase
DAD – diode array detector
DAP – days after planting
DD – day-degrees
DM – dry matter
DNA – deoxyribonucleic acid
e.g. – for example
ESI – electrospray ionization
ETC – electron transport chain
FM – fresh matter
H2O2 – hydrogen peroxide
HPLC – high performance liquid chromatography
HPS lamps – high pressure sodium discharge lamps
i.e. – that is
[M-H]- – deprotonated molecular ion
MeOH – methanol
mRNA – messenger ribonucleic acid
MS – mass spectrometry
m/z – mass-to-charge ratio
NADP+ – nicotinamide adenine dinucleotide phosphate
NIR – near infrared light
1O2 – singlet oxygen
O2.- - superoxide anion
PAL – phenylalanine ammonia lyase
PAR – photosynthetically active radiation
PPFD – photosynthetic photon flux density
PTFE - polyfluortetraethylene
ROS – reactive oxygen species
RT – retention time
SOD – superoxide dismutase

11.2 List of figures

11.3 List of tables

11.4 Diode array detector spectra of the investigated compounds

11.4.1 Quercetin and luteolin glycosides as well as caffeic acid derivatives

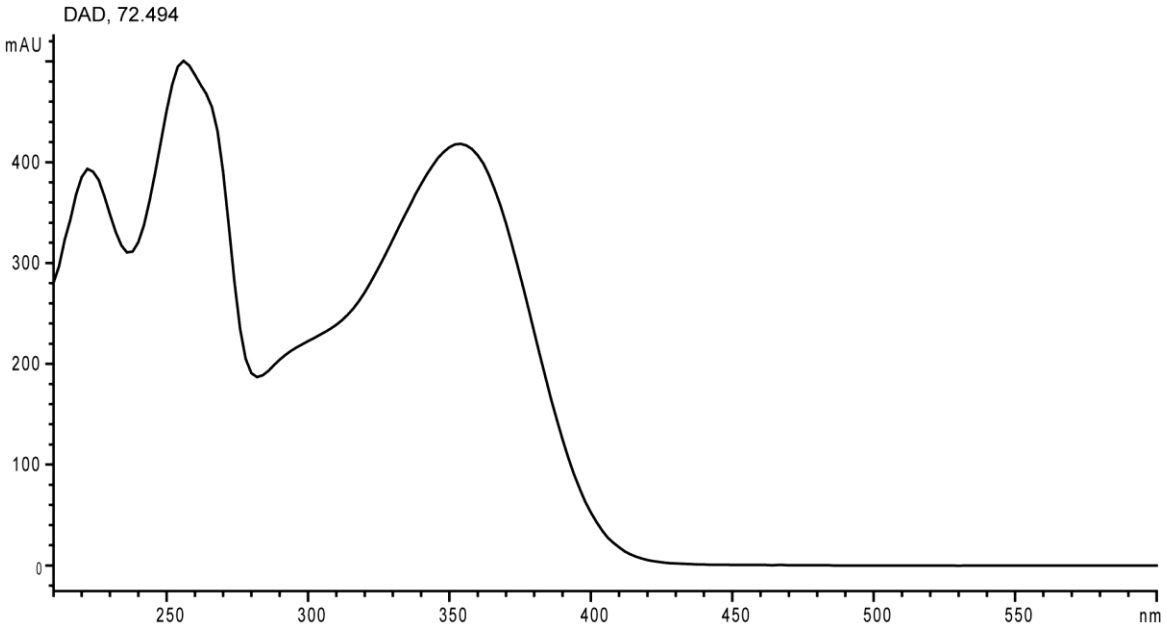

Figure 41: DAD absorption spectrum of quercetin-3-*O*-(6''-*O*-malonyl)-glucoside peak, retention time: 72.5 minutes.

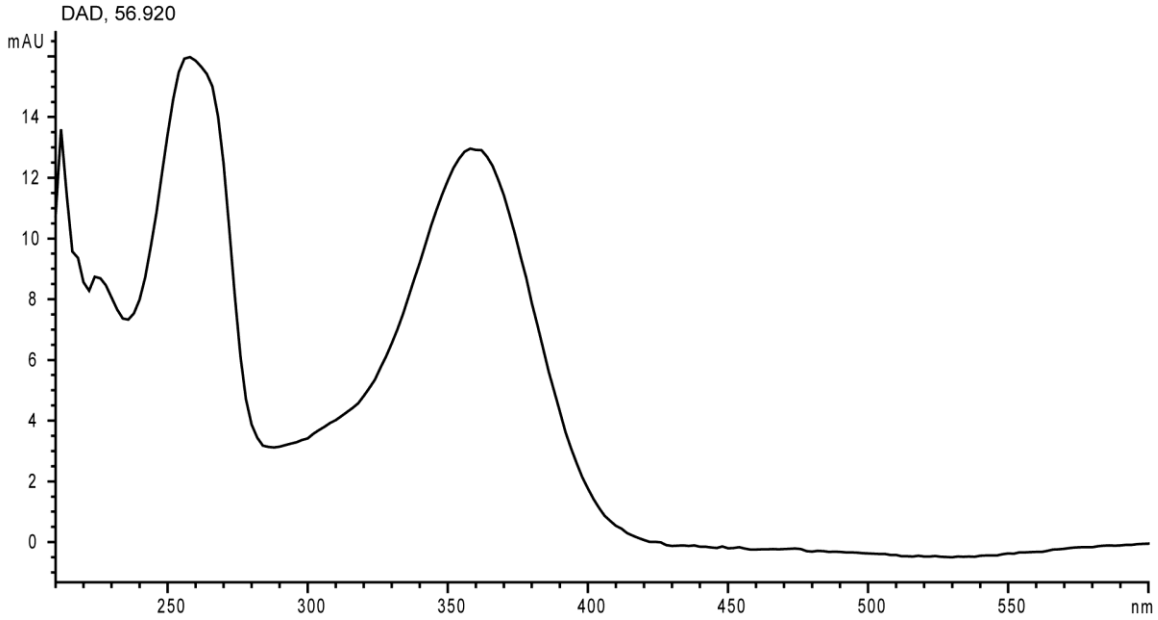

Figure 42: DAD absorption spectrum of quercetin-3-*O*-glucoside peak, retention time: 56.9 minutes.

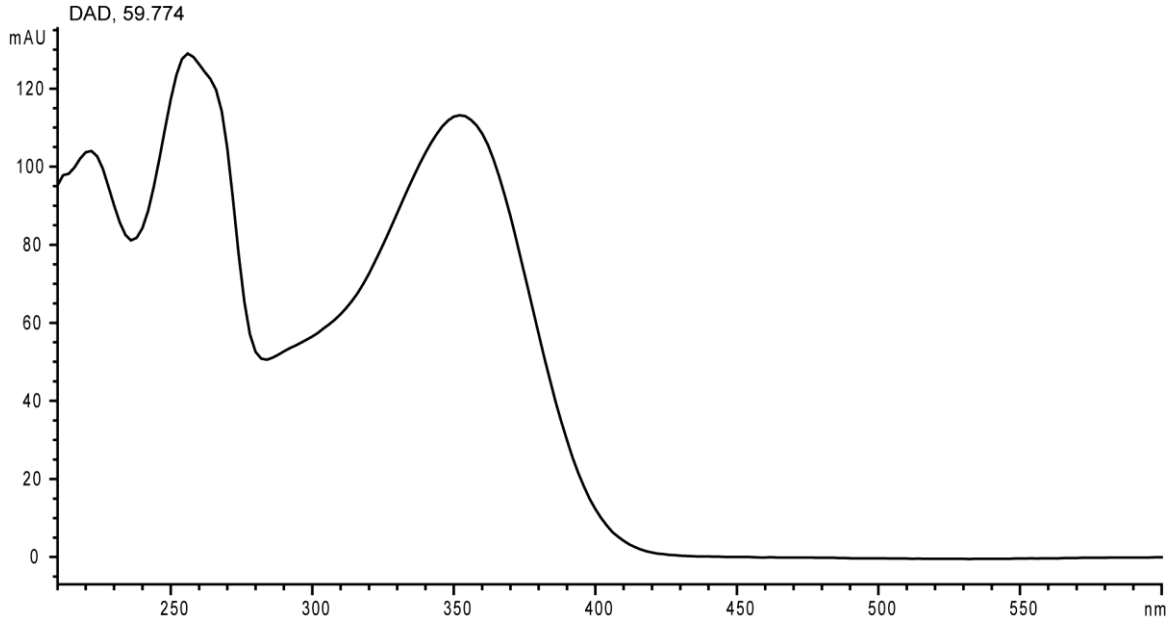

Figure 43: DAD absorption spectrum of quercetin-3-*O*-glucuronide/ luteolin-7-*O*-glucuronide peak, retention time: 59.8 minutes

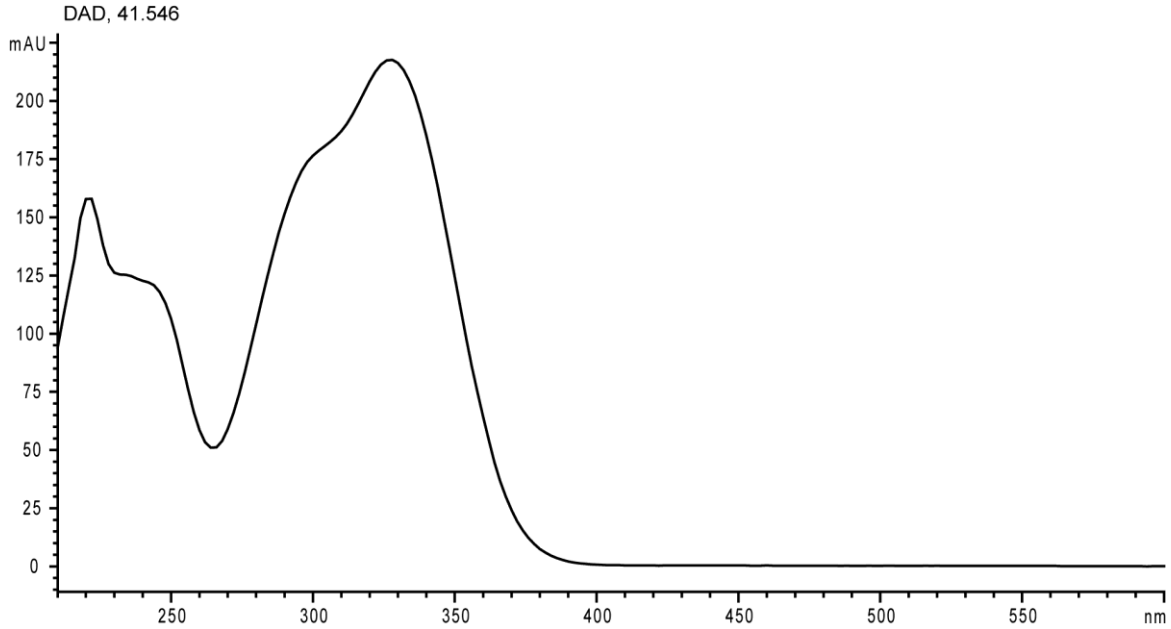

Figure 44: DAD absorption spectrum of chicoric acid peak, retention time: 41.6 minutes.

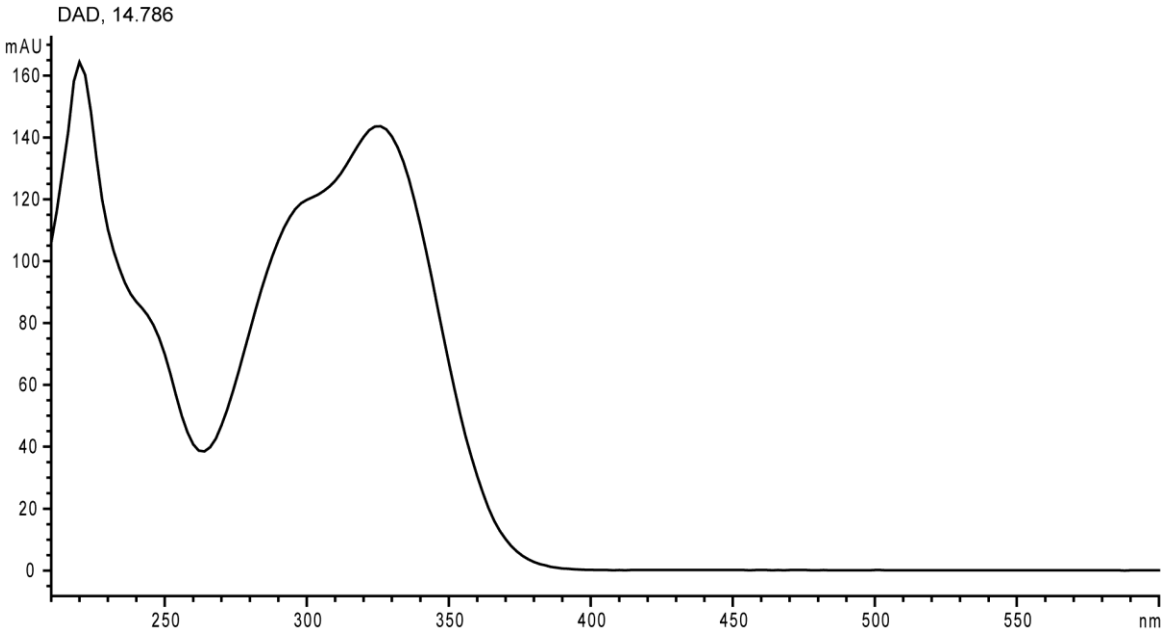

Figure 45: DAD absorption spectrum of chlorogenic acid peak, retention time: 14.2 minutes.

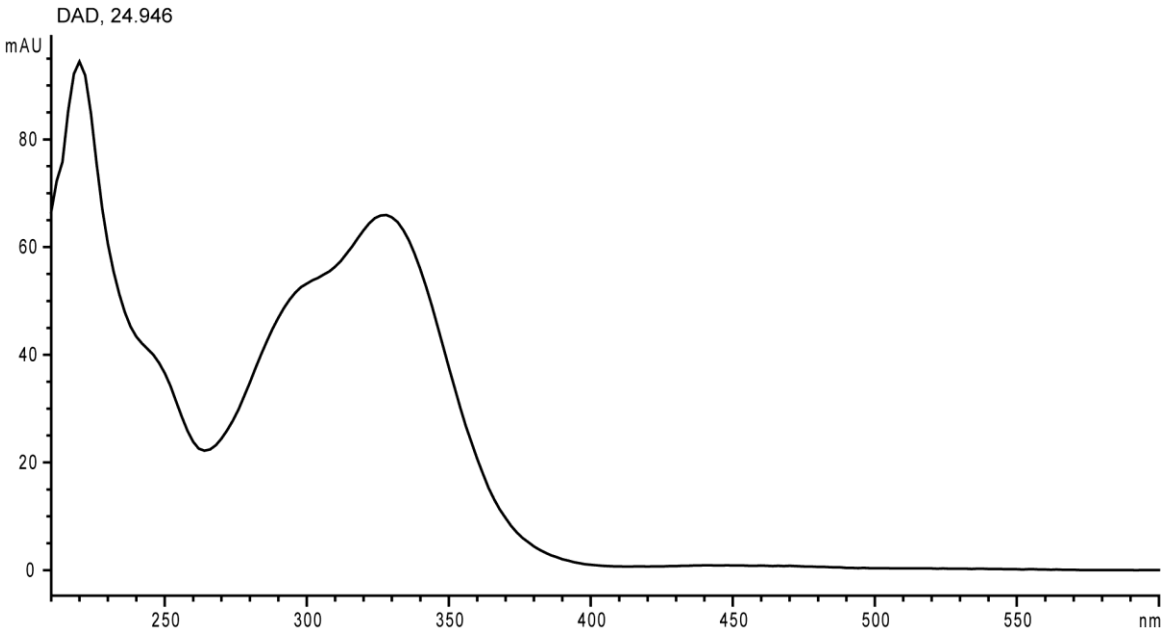

Figure 46: DAD absorption spectrum of caffeoylmalic acid peak, retention time: 25.0 minutes.

11.4.2 Cyanidin glycoside

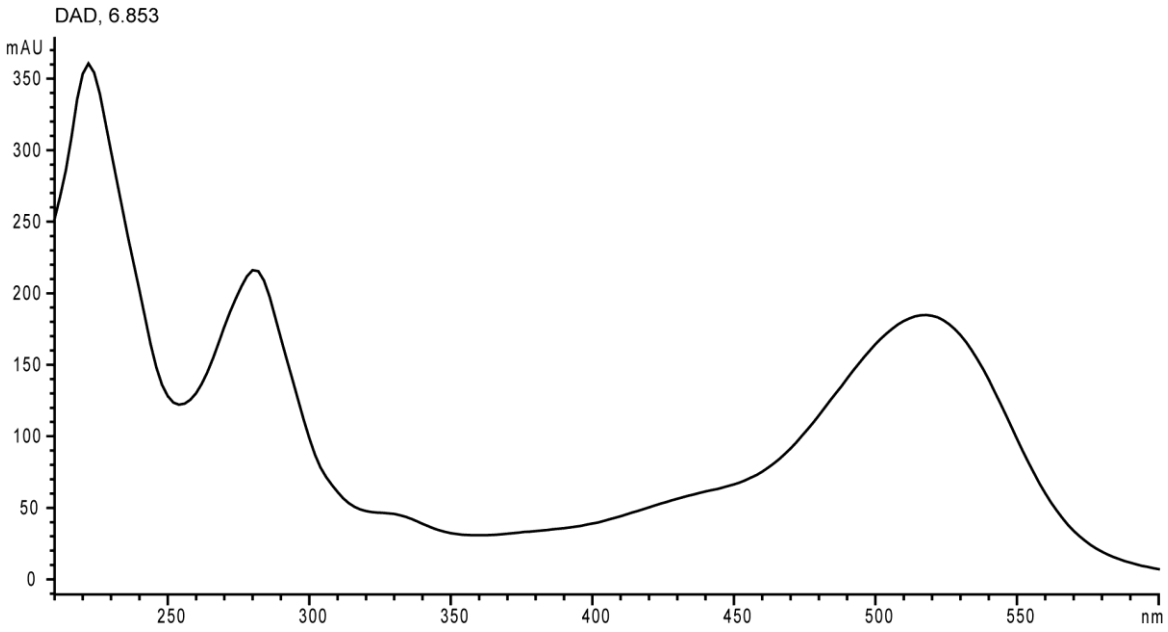

Figure 47: DAD absorption spectrum of anthocyanin peak at retention time of 6.9 minutes.

11.5 Radiation spectra in the climate chamber and in the greenhouse

Comparison of the spectra emitted by high pressure sodium lamps in the growth chamber and the solar spectrum (tab. 6, fig. 40, fig. 42) clearly displays differences. While the HPS lamps emphasize the orange wavebands, the sunlight spectrum covers the wavelengths of visible light much more evenly: Photons of these two ranges contribute in about equal shares to the solar spectrum, the ratio is about 10:1 in the HPS lamp spectrum (tab. 6). Hence, compared to natural daylight, there was an imbalance between red and blue wavebands in the HPS lamp spectrum.

Setting off the greenhouse glass transmission (fig. 41, tab. 6) against the sunlight spectrum (fig. 42, tab. 6) shows that wavebands in the visible and in the near infrared ranges are reduced uniformly, whereas UV radiation is dramatically reduced (51%, tab. 6, fig. 41). Thus, UV radiation provided in both experimental settings was very low. However, transmission of greenhouse glass for blue wavelengths is very good (fig. 41, tab. 6).

Table 6: HPS lamp spectrum (Philips SON-T 400 W, data by Maria Skoruppa) compared to solar irradiation spectrum, recorded in Ås, Norway (57.9°N) over the year (Hansen 1984), and transmittance of greenhouse glass cover (float glass; data by Dr. Burkhard von Elsner) and of the used shading nets (measured with JASCO V-670/ ILN-725; JASCO, Tokyo, Japan) depending on the respective wavelengths. Data is given in percent. Radiation categories are taken from Hansen (1984). UV = ultraviolet, NIR = near infrared.

Radiation category	Wavebands (nm)	HPS lamp spectrum (%)	Solar spectrum (%)	Greenhouse glass transmittance (%)	Shading net transmittance (%)
UV A + B	295 – 385	0.7	4 – 5	50.8	40.7
blue	385 – 495	5.9	14 – 16	89.5	45.0
green/ orange	495 – 630	62.6	16 – 19	89.9	46.7
red	630 – 695	9.0	7.5 – 8.5	87.8	41.8
NIR	695 – 2800	18.9[a]	58 – 51	83.8	56.2

[a] Dataset on HPS lamp spectrum only contains values up to 1078 nm

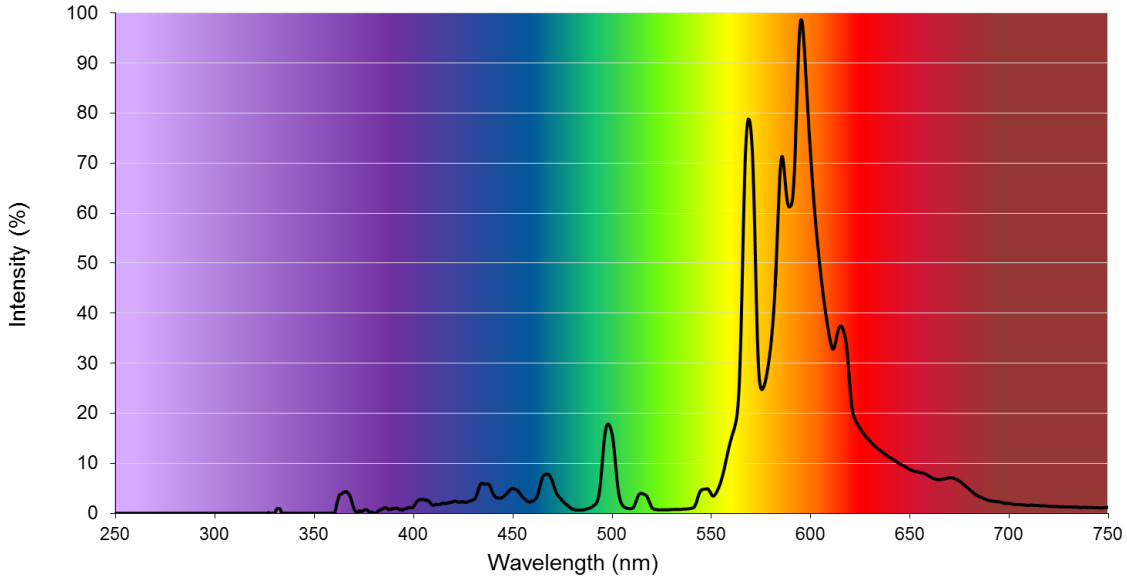

Figure 48: Radiation spectrum emitted by Philips SON-T high pressure sodium lamps in 104 cm distance (data provided by Maria Skoruppa, Leibniz-Institute for Vegetable and Ornamental Crops Grossbeeren)

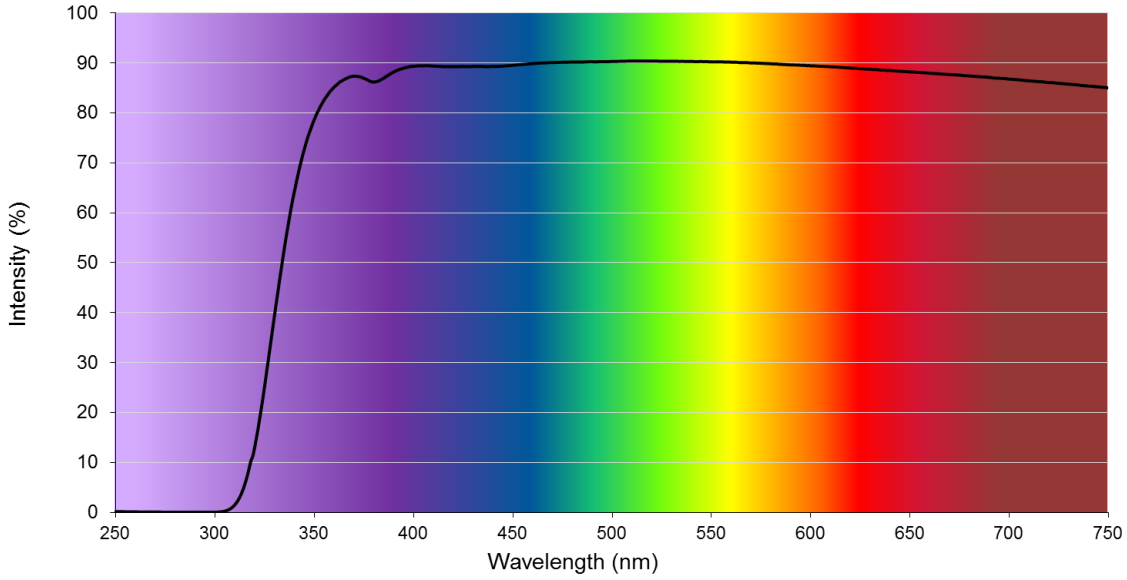

Figure 49: Transmission of radiation in percent by greenhouse glass cover (float glass) depending on wavelength (data was generously made available by Dr. Burkhard von Elsner, Leibniz University Hannover).

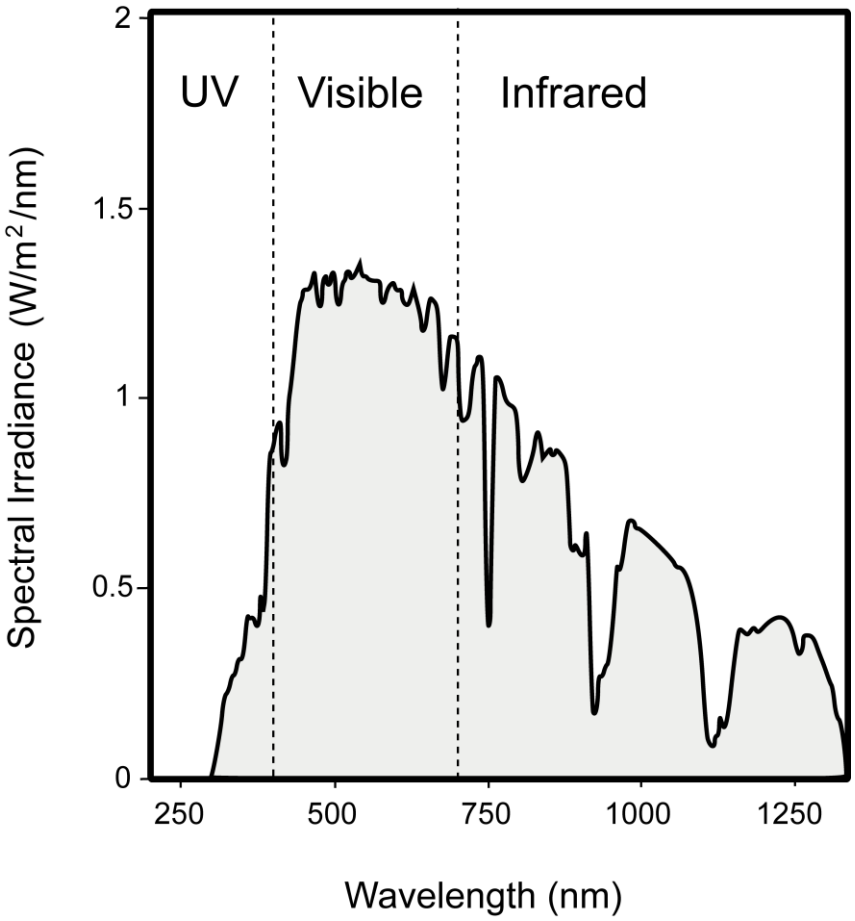

Figure 50: Solar irradiance at sea level (Robert A. Rohde, Wikimedia Commons, modified)