■ Methods—*invited article*

Analysis of phenolic compounds: which factors to consider?

Susanne Neugart, ORCID: 0000-0002-4574-6733
Leibniz-Institute of Vegetable and Ornamental Crops, Theodor-Echtermeyer-Weg 1, 14979 Grossbeeren, Germany
DOI: 10.19232/uv4pb.2017.1.13

Introduction

Phenolic compounds are of special interest as antioxidants and shielding compounds accumulated in response to ultraviolet-B radiation (UVB; λ = 280-315 nm) and other abiotic factors. The majority of phenolic compounds are based on phenolic acid or flavonoid aglycones (Schmidt et al. 2010a), but mainly occur in plants as glycosides (Calderon-Montano et al. 2011) and some of these compounds are acylated (Calderon-Montano et al. 2011; Schmidt et al. 2010b). Additionally, in case of flavanols, polymerization leads to tannins, also known as proanthocyanidins (Gadkari and Balaraman 2015). The compounds can vary from simple to highly complex structures, which makes their identification and quantification challenging.

To date, there are three main approaches to measure phenolic compound concentrations in plants.

1. The spectrophotometric measurements of total phenolic content, total flavonoid content, total flavonol content or others. However, this approach does not allow for the identification of single phenolic compounds within the extract. Moreover, to quantify content, standards like gallic acid are used that do not necessarily occur in the sample. A benefit is that the results are comparable to other data due to the intensive usage of this method worldwide, which is of high interest to compare plants and results of different labs.

2. The measurement of flavonoid aglycones after acid, alkaline or enzymatic hydrolysis of the flavonoid glycosides is best performed with high-pressure liquid chromatography (HPLC). A number of aglycone standards are already available, and thus, the identification and quantification of these compounds are both possible.

3. The identification and quantification of flavonoids glycosides require an analytical platform including HPLC coupled to a mass spectrometer (MS). For the identification of new compounds, nuclear magnetic resonance (NMR) would also be mandatory. This approach is mainly semi-quantitative due to the fewer standards that are currently available.

Of note is that there is so far no standard procedure for the measurement of phenolic compound profiles and concentrations in plants. However, the review of Julkunen-Tiitto et al. (2015) summarizes possible techniques to investigate the effect of UVB radiation on plant phenolics.

This article provides a comprehensive overview on how sampling, drying and storage as well as extraction and measurement affect the quantification of phenolic compounds in plants (Fig. 7.1). Furthermore, the need of

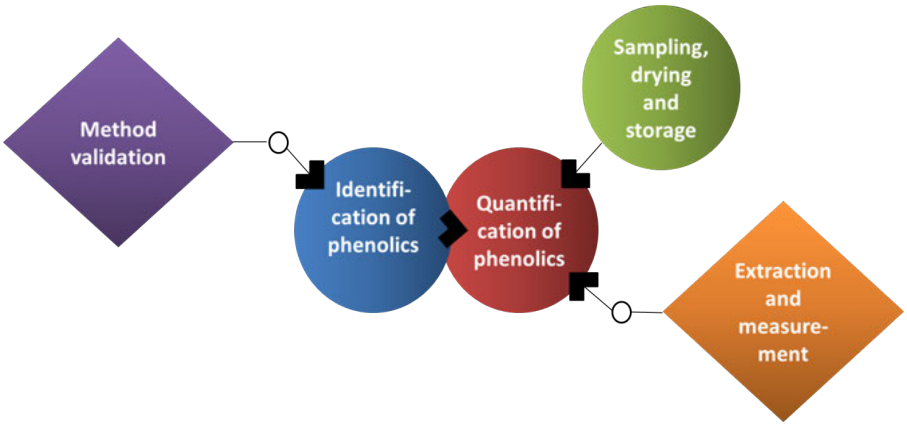

Figure 7.1: How sampling, drying and storage as well as extraction and measurement affect the quantification of phenolic compounds and the need of the method validation for the identification and quantification of phenolics.

method validation to be able to obtain reliable results regarding the identification and quantification of these compounds will be highlighted. Here, results from our research, done at the IGZ and TU Berlin, on kale's phenolic compound profile and concentrations will be used as examples (Neugart et al. 2013, 2014; Neugart, Kläring, et al. 2012; Neugart, Zietz, et al. 2012; Schmidt et al. 2010a,b; Zietz et al. 2010). The major benefit of using these experiments as examples is the possibility of comparing the same cultivar under different abiotic factors based on metabolite measurements obtained with the exact same validated method, including freeze-drying, extraction and measurement which would not be the case in a broad literature review.

This article does not provide a widely applicable standardized working protocol. Instead, it describes all the steps required for reliable quantification, highlighting the difficulties we encounter when comparing and interpreting phenolic compound measurements reported in the scientific literature, as there are enormous differences that may not only be dependent on the genotype and environment, but also depend on the various analytic approaches and protocols used in different labs. Below, we discuss the different possible sources of uncertainty at different steps of the quantification protocols and how we can control them.

Sampling

The sampling of the plants is a crucial step in measuring the concentration of phenolic compounds and should be considered carefully. Sampling protocols determine how the results of a study can be interpreted, and which conclusions can be validly drawn. The objective of the study and the desired range of validity of the results determine the design of the sampling protocol to be used. Plants' chemical composition is affected by both genotype and environment, while the amount of uncontrolled variation and avoidance of bias depend on the design of experiments, surveys and sampling protocols from an statistical point of view.

A valid approach for sampling the plants is to repeat the same experiment 3–4 times and generate independent results. This is the standard for climate chamber experiments requiring the use of multiple chambers or replication in time with random re-allocation of treatments to chambers and positions within chambers. In order that field experiments can generate truly interpretable data, the climate conditions should be monitored,

allowing the relationships between environmental variables and metabolite concentrations to be examined. In field experiments, broad validity will require replication in time and/or space at a scale matching the intended validity of the results: varying from replicate plots at one site and time—e.g. a randomized block design with at least 3–4 biological replicates–to temporal (years) and/or spatial (localities) replication—e.g. a regional or national network of field sites with replication over several years.

Generally, when comparing species or cultivars, the harvested plants or parts of plants should be grown and treated under the same conditions as far as possible to be able to obtain accurate results. More generally, random variation should be either controlled or quantified.

Other factors which can affect profiles and concentrations of phenolic compounds should be also considered: cultivar and genotype, developmental stage, plant organ, nutritional status of the plant, temperature and photosynthetically active radiation (PAR), UVB radiation, diurnal changes, as well as wounding. All these factors need to be considered when designing a sampling protocol and randomization applied to the sampling protocol so as to ensure that no bias is introduced. It is important to be aware that concentrations per unit dry matter can change rapidly both through fast synthesis, transformation or degradation of the metabolites under study, and by changes in the accumulation of other metabolites such as starch (e.g. starch concentration in leaves varies through the day as well as in response to any factor affecting photosynthesis or respiration—in the case of respiration, even after their collection, the dry mass of samples decreases, unless they are rapidly frozen or dried so as to stop metabolism.)

As a final consideration, data analysis should be performed taking into account what units in an experiment are true replicates and which ones are sub-samples within such replicates. In many cases valid analysis requires the use of nested designs to take into account the properties of the sampling scheme used.

Species or Cultivar

It is known that there is strong variation in phenolic compound profiles and concentration among species (Häkkinen and Törrönen 2000; Klepacka et al. 2011; Neugart et al. 2017, 2015; Wu et al. 2004). However, it is also now known that such variation exists between different cultivars of the same species. For example, various studies have shown that plants of different cultivars grown under the same conditions have remarkable differences in their phenolic compound concentration as described in the literature (Buendía et al. 2010; Castillo-Muñoz et al. 2010; Flanigan and Niemeyer 2014; Luo et al. 2013; Y.-W. Lv et al. 2011; Pérez-Gregorio et al. 2010; Wang et al. 2008; Zheng et al. 2012). For examples of kale, see sections Flavonoid Aglycones and Flavonoid Glycosides. If species or cultivars should be compared it is mandatory to do that in one experiment with the same environmental conditions regarding developmental stage, temperature, radiation and plant nutrition.

Developmental Stage

For sampling, plants should be at the same developmental stage, unless developmental effects are being studied. When interpreting results, one should take into account that environmental conditions, including the treatments under study, can affect the timing of plant development, such as reproductive induction. Moreover, variation of the phenolic compounds concentration with age has been described for several species (Edwards et al. 1997; Reifenrath and Müller 2007; Schoedl et al. 2012; Vogt and Gul 1994). As an additional example, in *Arabidopsis thaliana* and other plant species, an increase of anthocyan-

ins with age has been attributed to the higher concentration of reactive oxygen species in older plants (J. Huang et al. 2010; Kovinich et al. 2014; Stracke et al. 2010; Yonekura-Sakakibara et al. 2011). Data from one of our experiments, are used to exemplify how kale plants grown under consistent environmental conditions (250 µmol m^{-2} s^{-1} and 10°C) show a decrease in flavonoids with ongoing plant development (Fig. 7.2).

In addition to the plant's age, leaves at different positions and/or different ages within a single plant can also differ enormously in their phenolic compound profile and concentration. For example, in adult kale plants (12 weeks), the younger, light-green coloured leaves from the top of the plant contained higher concentrations of quercetin than the intermediate-aged or old leaves (Fig. 7.3), and similar results were found for strawberry fruits (Tsormpatsidis et al. 2011). Therefore, it is important to harvest parts of the plants that are at comparable developmental stage if one is to get meaningful and robust data. This does not necessarily mean a single developmental stage, but rather that samples from different individuals should be comparable in this respect in all cases—i.e. sampling of a single developmental stage of the plant or organ, multiple stages as separate samples, or as a pooled "stratified" sample.

Variation Within and Between Organs

Different plant organs vary in their flavonoid profile and concentration (El Morchid et al. 2014) and even within a single organ, variation has been found (e.g. Julkunen-Tiitto et al. 2015). As a further example, we found that the concentration of flavonoids in the midrib of kale leaves is at most 20% of what is found in the rest of the leaf, consistently accross different cultivars (Fig. 7.4). To minimize the effect on the results of differences between cultivars size of the leaves' midribs, one solution is to cut out the midrib where possible or sample the whole leaf and quantify the contribution of the midrib to each leaf's dry mass. Consequently, the harvested organs should be as equal as possible regarding developmental stage.

Nutritional Status of the Plants

Another factor affecting the phenolic compound concentration is the nutritional status of the plants. For example, several articles have described a negative correlation of phenolics, especially flavonoids, and nitrogen supply in the plants (Fallovo et al. 2011; Groenbaek et al. 2016, 2014; Han et al. 2010; Løvdal et al. 2010; Nguyen and Niemeyer 2008; K. M. Olsen et al. 2009; Strissel et al. 2005). We exemplify these effects, with data from one of our experiments on kale, where we observed that quercetin greatly decreased while kaempferol and isorhamnetin slightly decreased with increasing nitrogen fertilization (Fig. 7.5). However, even though other nutrients can also affect the concentration of phenolic compounds, they are less frequently discussed: e.g. sulphur fertilization can lead to a species-specific increase of phenolic acids in *Brassica rapa* subsp. *sylvestris* (De Pascale et al. 2007). Consequently, in pot experiments the same batch of well mixed soil should be used for all plants that are compared in an experiment, and pots assigned at random to different treatments. In field experiments the soil should be monitored for the nutrient status. Fertilizer applications during an experiment should be considered while interpreting the results and should be described in enough detail as part of the experimental methods.

Temperature and PAR

Temperature and photosynthetically active radiation (PAR) can affect the profile and concentration of phenolic compounds (Bernal et al. 2013; Chennupati et al. 2012; Mølmann et al. 2015; Mori et al. 2007; H. Olsen et al. 2009; Uleberg et al. 2012; Zandalinas et al. 2017). In

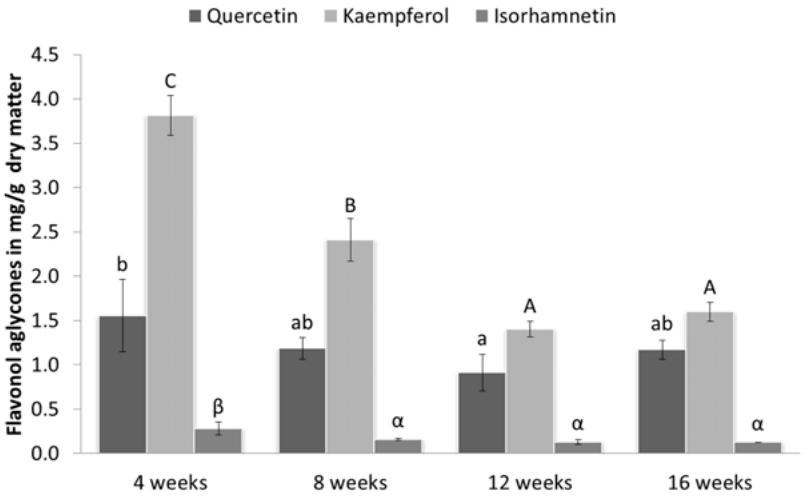

Figure 7.2: Flavonol aglycone concentrations of kale cv. 'Winterbor' dependent on the plant's developmental stage in plants grown at 10°C and 250 µmol/m² s. Different letters indicate significant differences between plants at different developmental stage for each flavonol aglycone ($p \leq 0.05$ by Tukey's HSD test). Each value represents the mean of three replicates ± SD. Susanne Neugart, unpublished data.

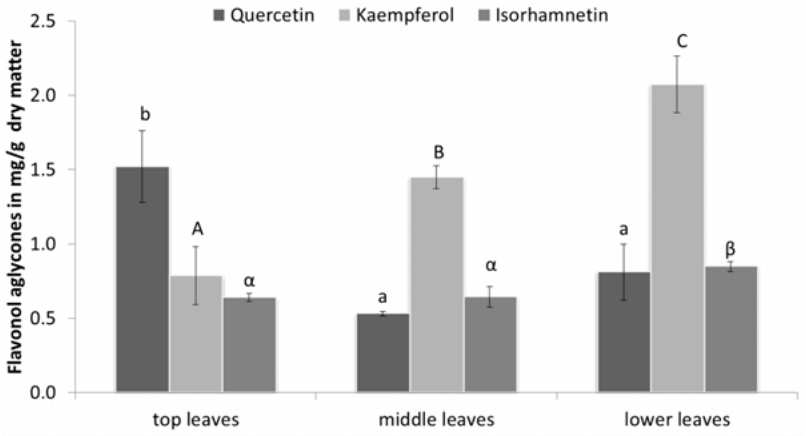

Figure 7.3: Flavonol aglycone concentrations of 12-week-old kale cv. 'Winterbor' dependent on the leaves' age in 12 week old plants grown at 10°C and 250 $\mu mol\,m^{-2}\,s^{-1}$. Different letters indicate significant differences between leaves of different age for each flavonol aglycone ($p \leq 0.05$ by Tukey's HSD test). Each value represents the mean of three replicates ± SD. Susanne Neugart, unpublished data

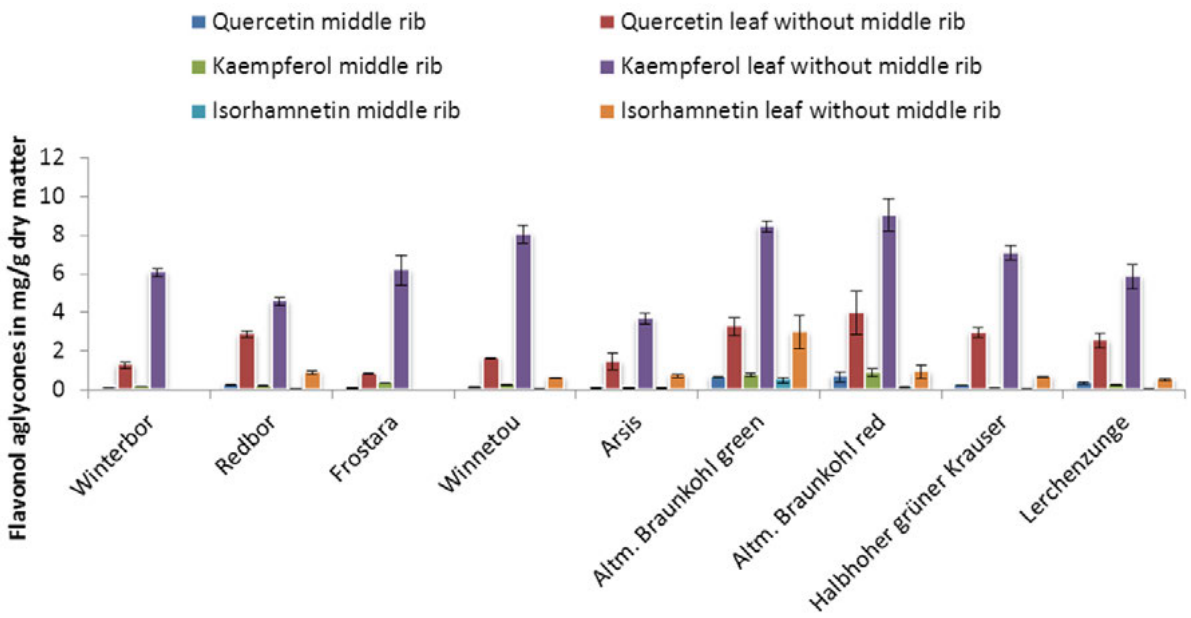

Figure 7.4: Flavonol aglycone concentrations of eight different kale cultivars grown in the field. The leaf was separated into midrib and leaf blade without midrib. The midrib contained remarkably lower concentrations of each flavonol aglycone. Susanne Neugart, unpublished data. For the statistical contrasts between the cultivars please refer to Schmidt et al. (2010).

several articles on kale, we have shown that the flavonoid glycoside concentrations are affected by temperature and photosynthetically active radiation (Neugart et al. 2013; Neugart, Kläring, et al. 2012; Neugart et al. 2016). Moreover, after acid hydrolysis, quercetin, kaempferol and isorhamnetin concentrations were observed to increase under low temperature (Fig. 7.6) whereas strong photosynthetically active radiation led to higher quercetin but lower kaempferol concentrations (Fig. 7.7). In more detail, blue and red light gained strong attention in horticulture production due to different effects on plant growth and metabolism (Demotes-Mainard et al. 2016; Huché-Thélier et al. 2016). While blue light induces mainly caffeic acid derivatives, quercetin and kaempferol glycosides as well as anthocyanins (Johkan et al. 2010; Nascimento et al. 2012; Siipola et al. 2014; Taulavuori et al. 2016) red light is not discussed as an inducer of phenolic compounds (Demotes-Mainard et al. 2016). However, synergistic effects of blue and red light are found (Johkan et al. 2010). In consequence, during sampling, one of the major challenges is to sample plants that have experienced the same conditions in temperature and radiation. Thus, one should harvest plants or plant parts from plants that are either from the middle of a block to avoid "border effects", or from one side of a row, or alternatively separately from each side of a row.

UVB Radiaition

Numerous researchers found effects of UVB radiation on the biosynthesis of phenolic compounds (Guidi et al. 2016; Jansen 2012; Lavola et al. 2013; Luis et al. 2007; Luthria et al. 2006; Z. Lv et al. 2013; Martínez-Lüscher et al. 2013; Morales et al. 2010; Nascimento et al. 2012; K. M. Olsen et al. 2009; Ryan et al. 1998; Suzuki et al. 2005; Tegelberg and Julkunen-Tiitto 2001). In more detail, UVB radiation is known to enhance the synthesis of B-Ring polyhydroxylated flavonoids such as quercetin and its glycosides (Becker et al.

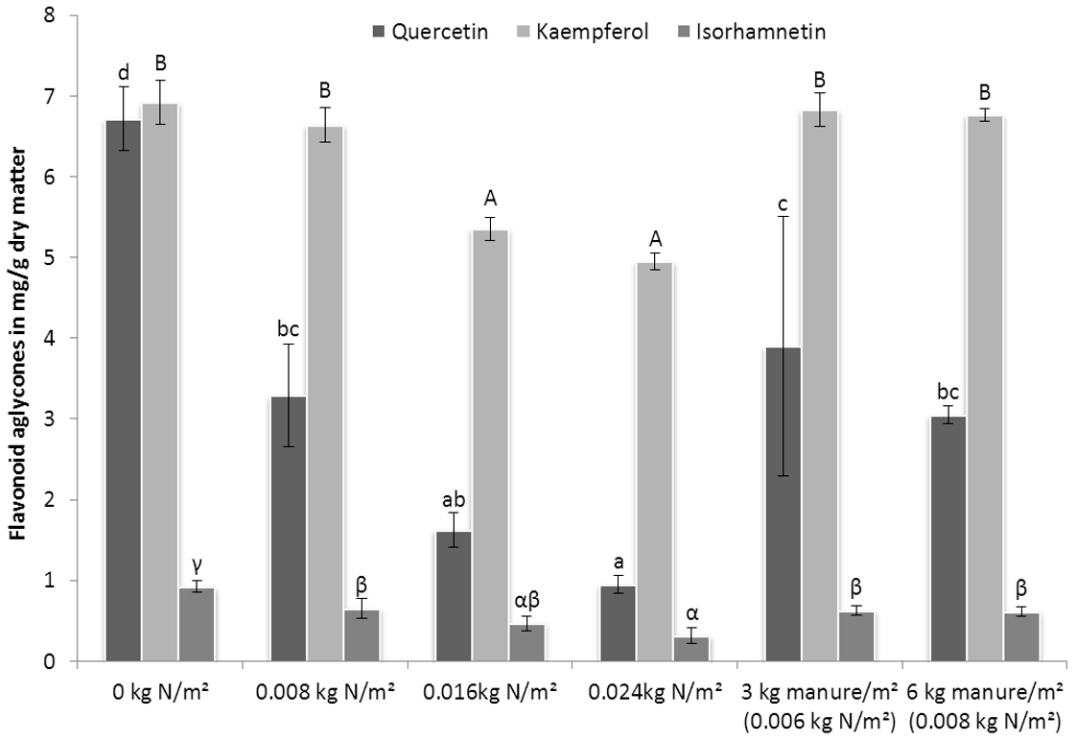

Figure 7.5: Flavonol aglycone concentrations of kale cv. 'Winterbor' dependent on the fertilization with mineral nitrogen (N) 0 to 0.024 kg/m² and organic manure 3 to 6 kg/m² (equals 0.006 to 0.008 kg N/m²). The plants were grown in the field. Different letters indicate significant differences between leaves of plants from different fertilization treatment for each flavonol aglycone ($p \leq 0.05$ by Tukey's HSD test). Each value represents the mean of three replicates ± SD.

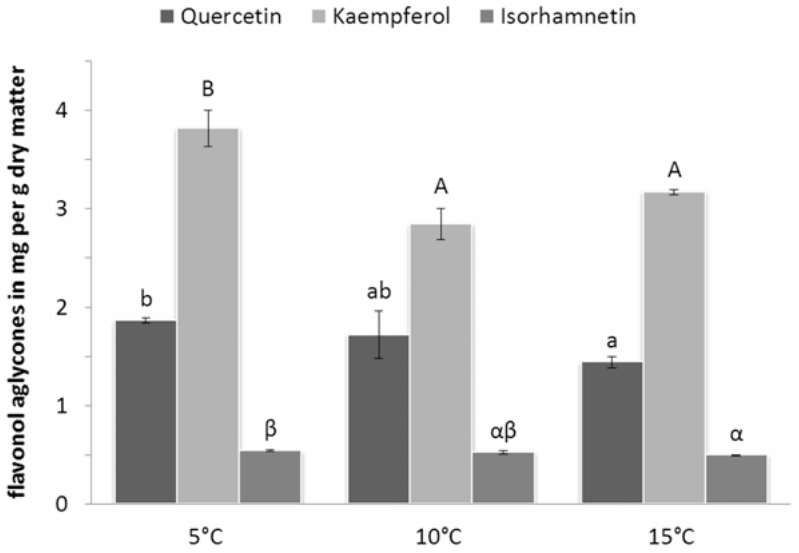

Figure 7.6: Flavonol aglycone concentrations of kale cv. 'Winterbor' dependent on the temperature in plants grown at 250 µmol/m² s. Different letters indicate significant differences between plants grown at different temperatures for each flavonol aglycone ($p \leq 0.05$ by Tukey's HSD test). Each value represents the mean of three replicates ± SD. Susanne Neugart, unpublished data

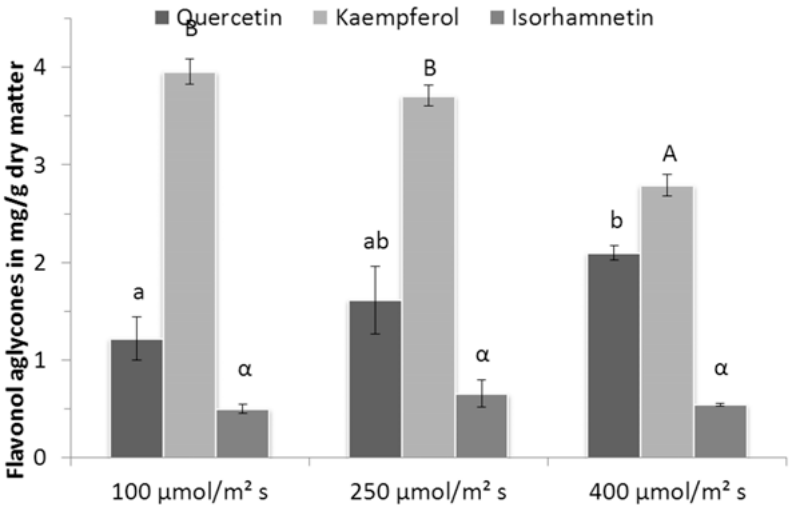

Figure 7.7: Flavonol aglycone concentrations in kale cv. 'Winterbor' plants grown at 10°C under different irradiances of photosynthetically active radiation. Different letters indicate significant differences in concentration between irradiance treatments, separately for each flavonol aglycone ($p \leq 0.05$ by Tukey's HSD test). Each value represents the mean of three replicates ± SD. Susanne Neugart, unpublished data.

2013; Bilger et al. 2007; Gliszczyńska-Świgło et al. 2007; Jaakola et al. 2004; Klem et al. 2015; Neugart, Zietz, et al. 2012; Tattini et al. 2005). Recently the interaction of UVB and temperature or water availability gained special interest (Bernal et al. 2015; Halac et al. 2014; Neugart et al. 2014). It is therefore extremely important to harvest plants or plant organs that have faced the same UVB conditions so either from the same side of the row in field experiments or of the same distance to artificial light sources in climate chambers. In case of UVB the exact measurement and monitoring of the UVB physiological quantities and the biological effective doses of UVB is mandatory for the interpretation of the data.

Diurnal Changes

Besides the accumulation of phenolic compounds as a response to environmental changes there is evidence that some plants exhibit diurnal changes in their flavonoid profiles and concentrations (reviewed by Julkunen-Tiitto et al. 2015). Therefore, the harvest should be as fast as possible and controls for each time point that plants are harvested (day or hour) should be taken if more than one harvest is necessary for the experiment.

Wounding

Sometimes sampling leads to wounding of the plants or single leaves. In such cases, the samples should be frozen in liquid nitrogen immediately to minimize any reaction caused by wounding even though that might need hours or even days (Surjadinata and Cisneros-Zevallos 2012). Sequential sampling of organs from the same individual plants should be avoided unless done within a very short length of time, such as a few minutes. One should also consider whether earlier, even non-destructive, measurements could have affected the organ or plant being sampled. In the case of wounding by herbivores, one should take into account how this relates to the objectives of the study, and if appropriate avoid sampling damaged plants, and in all cases taking note of any abnormal fea-

tures of individual plants or organs sampled.

In conclusion, the design of a sampling protocol must take into account the aims of the study. In climate chamber experiments it is simple to have defined conditions and change exclusively one or two factors. However, it is much more complicated in field experiments. It is recommended to sample plants at the same developmental stage and that were grown under the same conditions regarding exposure to various abiotic factors such as temperature and radiation. If different developmental stages or abiotic conditions are part of the experiment, these differences should be accurately measured, e.g. temperature and irradiance as well as leaf number and size. If plants are sampled from different growing locations, it can be useful to have soil samples to also allow for the assessment of the plants' nutritional status. All of the above components that make up the sampling method affect what conclusions can be validly drawn from the measurements. If single plants or leaves are sampled the biological variability among them can be directly estimated. If pooled samples are used, such estimates of variability need to be estimated indirectly. Relying on a minimum of 5 samples facilitates the detection of outliers. From the statistical point of view, what matters is the number of true replicates, which usually are neither individual plants nor leaves. Pooling of samples from different plant parts discards information but can be used if the mass of individual samples is too low. A non-random sampling sequence can be a source of bias. The order in which each plant is sampled should always be random, within blocks if present in the design of the experiment. Randomization should ensure that the sampling of individuals from different treatments is interspersed in time. The comparability and reproducibility of results reported is much improved when authors describe the sampling protocol in detail.

Drying and Storage

A comprehensive summary of drying methods and storage conditions is provided by Julkunen-Tiitto et al. (2015). In general, samples should be as dry as possible which is easily achieved by freeze-drying. If the humidity of the sample is less than 3%, samples can be stored at room temperature (Pérez-Gregorio et al. 2010). Additionally, the samples should be stored in desiccators if needed e.g. in summer or tropical climate. Otherwise the samples can be stored at -20°C to avoid the effect of humidity (Syamaladevi et al. 2011). This is the same for anthocyanins (Pérez-Gregorio et al. 2010; Syamaladevi et al. 2011). It is known that, in solution, quercetin readily photodegrades (Dall'Acqua et al. 2012), while degrading at a slower rate in dry samples. Nevertheless, samples should be analysed as soon as possible to minimize the effect of degradation on metabolite quantification.

Extraction and Measurement

Currently, there are numerous methods for extracting phenolic compounds (reviewed by Julkunen-Tiitto et al. 2015). However, a major challenge still remains in the identification and quantification of phenolic compounds in the plant matrix. In detail, the end measurement is strongly influenced by the extraction method, e.g. acid hydrolysis vs. methanolic extraction. It is important to always remember that the size of particles in a sample affects extraction efficiency—the smaller the size, the better. The second crucial point that should be taken into account is that when using a portion of a larger sample for an extraction, any lack of homogeneity in the sample compromises quantification through decreased accuracy (see section Reproducibility on page 50). If samples are difficult to grind, or inhomogeneous, it is a good trick-of-the-trade to increase the sample mass that is extracted to 1 g or more

and proportionally increase the volume of the extraction solution. The extraction generally is more efficient at higher temperatures (up to 40°C) and with longer extraction time (see section Optimization of the Extraction on page 49). If a large number of plants should be compared, it can be of use to be less specific and measure the total phenolic content (TPC) or the flavonoid aglycones and other phenolic compounds' aglycones. If the response of one plant to a biotic or abiotic factor is investigated, it can be helpful to discuss structure-activity-relationships based on a very detailed analysis of flavonoid glycosides and other phenolic compounds' glycosides.

Below is a summary of extraction methods and measurement of phenolic compounds we have previously successfully used for several species after optimization for each species. Results from our studies on kale are used to exemplify what kind of data can be generated and how these can be interpreted.

Total Phenolic Content

This approach is a fast method to quantify the total phenolic content, or TPC (for more details on this and other assays see Julkunen-Tiitto et al. 2015) in plants but was originally developed to measure proteins (Lowry et al. 1951). It yields a single measured value per sample analysed, which is assumed to behave as an approximate joint quantifier for a large group metabolites. The potential interaction with proteins is one of the disadvantages of this method. Some researchers also consider vitamin C and other antioxidants as these compounds can also react with the Folin-Ciocalteu regent and influence the results. However, the method has the advantage that different plants can be compared due to the reference gallic acid. Additionally correlations to the antioxidant activity are often also measured. Plants differ in their phenolic profiles and this might lead to over- or underestimation of the real concentrations of these phenolic compounds. Consequently, when treatments affect the phenolic profile, or when genotypes differ in their metabolite composition, bias in TPC measurements can be introduced by the use of this method. One solution is to additionally measure the plant's main phenolic compound as a standard reference and then to quantify the TPC based on this. If the antioxidant activity is to be correlated to the total phenolic content, it is recommended to measure other antioxidants of relevance in the plant as well e.g. vitamin C or carotenoids. Nevertheless, different extraction methods regarding kind of solvent, volume of solvent, extraction time, extraction temperature and other possible influencing factors such as pH can affect the quantification of the TPC, and therefore, should be optimised in order to accurately validate the different methods used. The same is true for other chemical reactions often used to estimate total flavonoid or anthocyanin content. Note that the method for the extraction and measurement of phenolic compounds needs to be validated see section Method Validation on page 46.

In kale, we analysed the TPC using the following method published in Zietz et al. (2010). For extraction, 2 g of ground sample were dissolved in 25 ml of 62.5% aqueous methanol and stirred at 500 rpm for 1 h. The mixture was then filtered and aliquots were used for further analysis. The total phenolic content TPC of kale extracts was determined using the Folin-Ciocalteu colourimetric method. In brief, 400 µl of a 20-fold dilution of each extract was mixed with 2.5 ml of distilled water, 1 ml of Na_2CO_3 (7.5% w/v) and 100 µl of Folin-Ciocalteu reagent. The thoroughly mixed solution was incubated at 35°C for 15 min. After the solution had cooled to room temperature, the absorbance was measured at $\lambda = 736$ nm (SPECORD 40, Analytik Jena AG, Jena, Germany). The results were expressed as millimoles of gallic acid equivalents per gram of dry matter (mmol GAE/g dry matter). All extracts were ana-

lysed in duplicate. In the eight investigated kale cultivars the gallic acid equivalent concentration (GAE) ranged between 0.18 and 0.31 mmol/g dry matter. The genotypic variation shows that especially the traditional, old cultivars 'Altmärker Braunkohl', 'Halbhoher gruner Krauser' and 'Lerchenzunge' as well as the red hybrid 'Redbor' are characterized by relatively high TPC, while the cultivars 'Winterbor', 'Frostara', 'Winnetou' and 'Arsis' have lower values. Those genotypes investigated in our study had higher TPC values than those found by other researchers, which ranged from 0.08 to 0.11 mmol GAE/g dry matter (Hagen et al. 2009; Heimler et al. 2006; H. Olsen et al. 2009). This difference might result from different extraction efficiency (see section Optimization of the Extraction on page 49).

Flavonoids as Flavonol Aglycones

As several flavonoids present in a given sample may share the same aglycone, and differ only in the attached residues, removing these residues decreases the number of compounds remaining in the extract. This is achieved through de-acylation and de-glycosylation of flavonoid glycosides to flavonoid aglycones. The advantage of this method is the smaller number of compounds being quantified. Of these aglycones, many are available as reference standards, which allows to accurately identify phenolic aglycones with HPLC by direct comparison against such reference standards. This identification can then be verified by mass spectrometry (then MS grade solutions for extraction solvents and measurement eluents are mandatory). Quantification of each peak compared to a standard reference is possible, i.e. the samples are measured and the peak areas under the curve for the aglycones from both samples and standards are computed. Calibration curves can then be produced with a minimum of four known concentrations of pure aglycones (see section Calibration Curves on page 51). The peak areas of the calibration curves should be in the same range as the peak areas of the samples. With the help of the calibration curves and the dilution factors (during extraction and measurement of the samples), the areas of the samples can be re-expressed as concentrations. Whether to give the concentrations in dry matter or fresh matter depends on the scientific question being addressed. For example, if the treatment of the plants or the general plant development leads to morphological changes, it is recommended to work with concentrations in dry matter due to a different water content expected.

In kale, we analysed the flavonol aglycones quercetin, kaempferol and isorhamnetin using the following method published in (Schmidt et al. 2010a). A lyophilized kale sample (0.5 g) was hydrolysed with 50% aqueous methanol and 1.6 M HCl in double determination experiments. After refluxing at 90°C for 2 h, the extract was cooled down to room temperature, adjusted to 100 ml and then sonicated for 5 min. After which, the extract was filtered through a 0.45 µm PTFE filter for HPLC analysis. With this method, phenolic acids and anthocyanidins can be measured. However, higher concentrations of HCl are needed to measure anthocyanidin concentrations (Merken et al. 2001). The extracts were separated on a Prodigy (ODS 3, 150x3.0 mm, 5 µm, particle size 100 Å) column (Phenomenex, Aschaffenburg, Germany) with a security guard C18 (ODS 3, 4 3.0 mm, 5 lm, 100 Å), at a temperature of 25°C using a water/acetonitrile gradient. Solvent A consisted of 99.5% water and 0.5% acetic acid; solvent B contained 100% acetonitrile. The following gradient was used for eluent B: 30–35% (0–5 min), 35–39% (5–17 min), 39–90% (17–21 min), 90% isocratic (21–26 min), 90–30% (26–29 min), 30% isocratic (29–34 min). Flow was performed using 0.3 ml min^{-1}, and the measured detector wavelength was $\lambda = 370$ nm. The standards dihydroquercetin, kaempferol and isorham-

netin (Carl Roth GmbH, Karlsruhe, Germany) were used to obtain an external calibration curve in the range of 0.01–10 mg/100 ml. The total flavonol concentration was calculated as the sum of the concentration of the individual flavonol aglycones quercetin, kaempferol and isorhamnetin. Quercetin, kaempferol and isorhamnetin in kale (Fig. 7.8) were identified by comparison to standards (Fig. 7.9) as deprotonated molecular ions and characteristic mass fragment ions by HPLC–DAD–ESI–MS2 with an ion trap mass spectrometer. The mass optimization was performed for quercetin [M-H]- m/z 301. The ESI source potential on capillary was 3.5 kV. The declustering voltage was -40 V and the focusing voltage was 153 V. The automated collision energy was 1 V (30–200%).

In eight kale cultivars including hybrid and traditional cultivars, high concentrations of the flavonol aglycones kaempferol and quercetin, followed by isorhamnetin were identified and quantified by comparison to standards. The total concentration of these flavonol aglycones was between 6.0 and 14.8 mg/g dry matter, which corresponds to 97.4–298.5 mg / 100 g fresh matter (Schmidt et al. 2010a). The genotypic variation revealed that traditional, old cultivars 'Altmärker Braunkohl', 'Halbhoher grüner Krauser' and 'Lerchenzunge' are characterized by relatively high flavonoid concentrations, while lower flavonoid concentrations were found in the hybrids 'Arsis' and 'Winterbor', as well as in the cv. 'Frostara'. Comparable concentrations to our results were also determined by (Z. Huang et al. 2007) in curly kale (*Brassica oleracea* var. *acephala*), with 90.5, 31.8 and 23.6 mg/100 g fm for kaempferol, quercetin and isorhamnetin, respectively. Furthermore, similar quercetin concentrations (7.7-24.4 mg/100 g fm) were detected in curly kale, but the kaempferol concentrations were much lower (21–47 mg/100 g fm) compared to our investigated cultivars, whereas isorhamnetin was not detected in these kale varieties (Hertog et al. 1992; Zhang et al. 2003) underlining the effect of strong photosynthetically active radiation during plant growth (Zhang et al. 2003) see section Temperature and Radiation on page 34. Note that phenolic acids and anthocyanindins were not investigated in these kale samples.

Flavonoids as Flavonol Glycosides

In contrast to methods described in the previous two sections, here the aim is to quantify the individual flavonoids as they are in the plant. With this method, one can investigate detailed structure-activity relationships and it is also the most precise approach to identifying phenolic compound profile and concentration. However, the reliable identification of these compounds is complex and time-consuming. To start with, an HPLC instrument coupled to a mass spectrometer (MS) as detector is needed. For precise structural determination other methods (such as nuclear magnetic resonance, i.e. NMR) are needed, e.g. to distinguish between isomers differing only in the position where the same substituent is bound. Very sophisticated MS methodology such as ion mobility could be useful for that purpose as well. Julkunen-Tiitto et al. (2015) summarize which extraction solvents, columns, eluents and wavelength as well as mass spectrometric parameters and NMR approaches can be used in the identification and quantification of flavonoids.

Due to the small number of available reference standards for complex glycosylated and acylated phenolic compounds, often only a semi-quantitative quantification based on related standards is possible. Further, it is not common to calculate response factors since reference standards are missing that would be required to do that. Generally, the quantification works as described for the flavonoid aglycones, but with one standard being used for different glycosides, e.g. quercetin-3-glucoside for several quercetin glycosides.

In kale, we analysed the flavonol glycosides

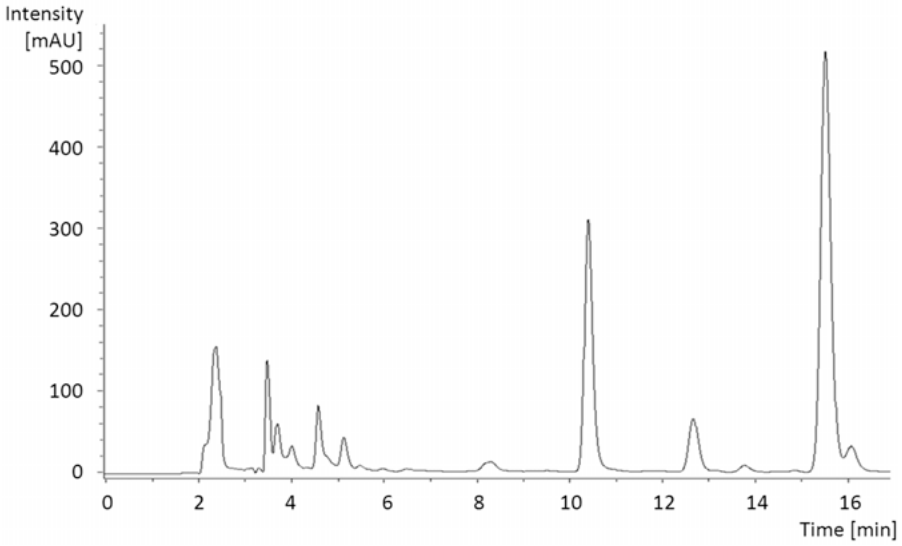

Figure 7.8: Cromatogram of flavonol aglycones in kale cv 'Winterbor' after acid hydrolysis at λ = 370 nm. Susanne Neugart, unpublished data.

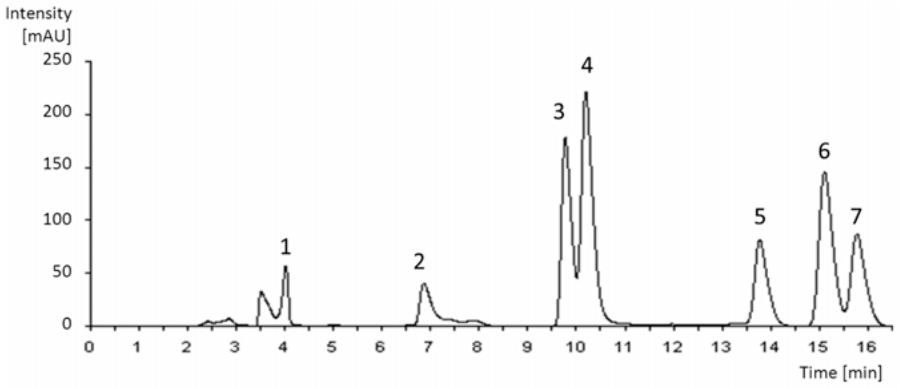

Figure 7.9: Chromatogram of a standard flavonoid glycoside and aglycone mixture at λ = 370 nm: 1-quercetin-3-rutinoside, 2-myricetin, 3-luteolin, 4-quercetin, 5-apigenin, 6-kaempferol, 7-isorhamnetin. Susanne Neugart, unpublished data.

and hydroxycinnamic acid derivatives using the following method published in (Schmidt et al. 2010b). Lyophilized kale (0.5 g) was extracted with 15 ml of 60% aqueous methanol on a magnetic stirrer plate for 1.5 h in a double determination approach. The extract was filtered through a fluted filter and subsequently evaporated to dryness. The residue was dissolved in 5 ml of distilled water and then filtered through a cellulose-mixed ether-membrane (CME) filter for HPLC analysis.

A modification for smaller volumes has been recently published in (Neugart et al. 2017). Lyophilized, ground plant material (0.02 g) was extracted with 600 µl of 60% aqueous methanol on a magnetic stirrer plate for 40 min at 20°C. The extract was centrifuged at 4500 rpm for 10 min at the same temperature and the supernatant was collected in a reaction tube. This process was repeated twice with 300 µl of 60% aqueous methanol for 20 min and 10 min, respectively. The three corresponding supernatants were then pooled. The extract was subsequently evaporated until it was dry and was then sus-

pended in 200 µl of 10% aqueous methanol. The extract was centrifuged at 3000 rpm for 5 min at 20°C through a Corning® Costar® Spin-X® plastic centrifuge tube filter (Sigma Aldrich Chemical Co., St. Louis, MO, USA) for HPLC analysis. Each extraction was carried out in duplicate. This method can also be used for hydroxycinnamic acid derivatives and proanthocynaidins. However, to date, no method validation for these compounds has been performed. Finally to extract anthocyanins, acidified methanol (0.1% v/v formic acid) is commonly used to stabilize the cations (H. Olsen et al. 2010). The flavonol glycosides were analysed using a Prodigy (ODS 3, 150 3.0 mm, 5 lm, 100 Å) column (Phenomenex, Aschaffenburg, Germany) with a security guard C18 (ODS 3, 4 3.0 mm, 5 lm, 100 Å) at a temperature of 25°C using a water/acetonitrile gradient. Solvent A consisted of 99.5% water and 0.5% acetic acid; solvent B contained 100% acetonitrile. The following gradient was used for eluent B (100 % acetonitrile) at a temperature of 30°C: 5–7% (0–12 min), 7–9% (12–25 min), 9–12% (25–45 min), 12–15 % (45–100 min), 15% isocratic (100–150 min), 15–50 % (150–155 min), 50 % isocratic (155–165 min), 50–5% (165–170 min), 5% isocratic (170–175 min). The flow was performed using 0.4 ml min^{-1}, and the measured detector wavelength for the quantification was set at $\lambda = 370$ nm for non-acylated flavonol glycosides and $\lambda = 330$ nm for acylated flavonol glycosides. The standards quercetin-3-O-glucoside and the corresponding 3-O-glucosides of kaempferol and isorhamnetin (Carl Roth GmbH, Karlsruhe, Germany) were used in a semi-quantitative approach to obtain an external calibration curve in the range of 0.1–10 mg/100 ml. Mass optimization for the ion optics of the mass spectrometer was performed for quercetin m/z 301 for the low mass flavonol glycosides. In addition, due to the lack of standards, arbitrary m/z 1000 was used as the target mass in auto-mode to include higher mass fragments for higher mass flavonol glycosides. The ESI source potential on capillary was 3.5 kV. The declustering voltage was -40 V and the focusing voltage was 153 V at mass optimization m/z 301 and 200 V at mass optimization m/z 1000. The automated collision energy was 1 V (30–200%). The MSn experiments were performed in auto- or manual mode up to MS4 in a scan from m/z 200 to 2000. Note that anthocyanins were not investigated in these kale samples. In kale, 71 flavonol glycosides have been tentatively identified by HPLC-DAD-MSn. Of these, 27 non-acylated, 30 monoacylated and 14 diacylated glycosides have been found based on the flavonol aglycones quercetin, kaempferol and isorhamnetin. Seven of these 71 compounds have been further identified with NMR in a previous study (Fiol et al. 2012). The main flavonol glycosides in kale are non-acylated and monoacylated quercetin and kaempferol glucosides, with the majority of flavonol glucosides being acylated with hydroxycinnamic acids. A presentation of selected quercetin and kaempferol glycosides is depicted in Fig. 7.10. Of the non-acylated (Fig. 7.10-A) and monoacylated (Fig. 7.10-B) compounds, the kaempferol glycosides were in higher concentration in the plants and traditional cultivars had higher concentrations than the hybrid cultivars-except for the cultivar 'Redbor'. Interestingly, this is different in the complex diacylated tetraglycosides (Fig. 7.10-C) which are in high concentrations only in cultivar 'Redbor'. Finally, our results on the identification and quantification of flavonoid glycosides in kale are supported by findings of other groups (Ferreres, Fernandes, Oliveira, et al. 2009; Lin and Harnly 2009; H. Olsen et al. 2009). Of note is that the precision had a variation coefficient up to 8% and the accuracy has a variation coefficient of up to 8% for the main phenolic compounds and up to 20% for the minor compounds which is higher than that for flavonol aglycones in kale see section Reproducibility (precision and accuracy) on page 50.

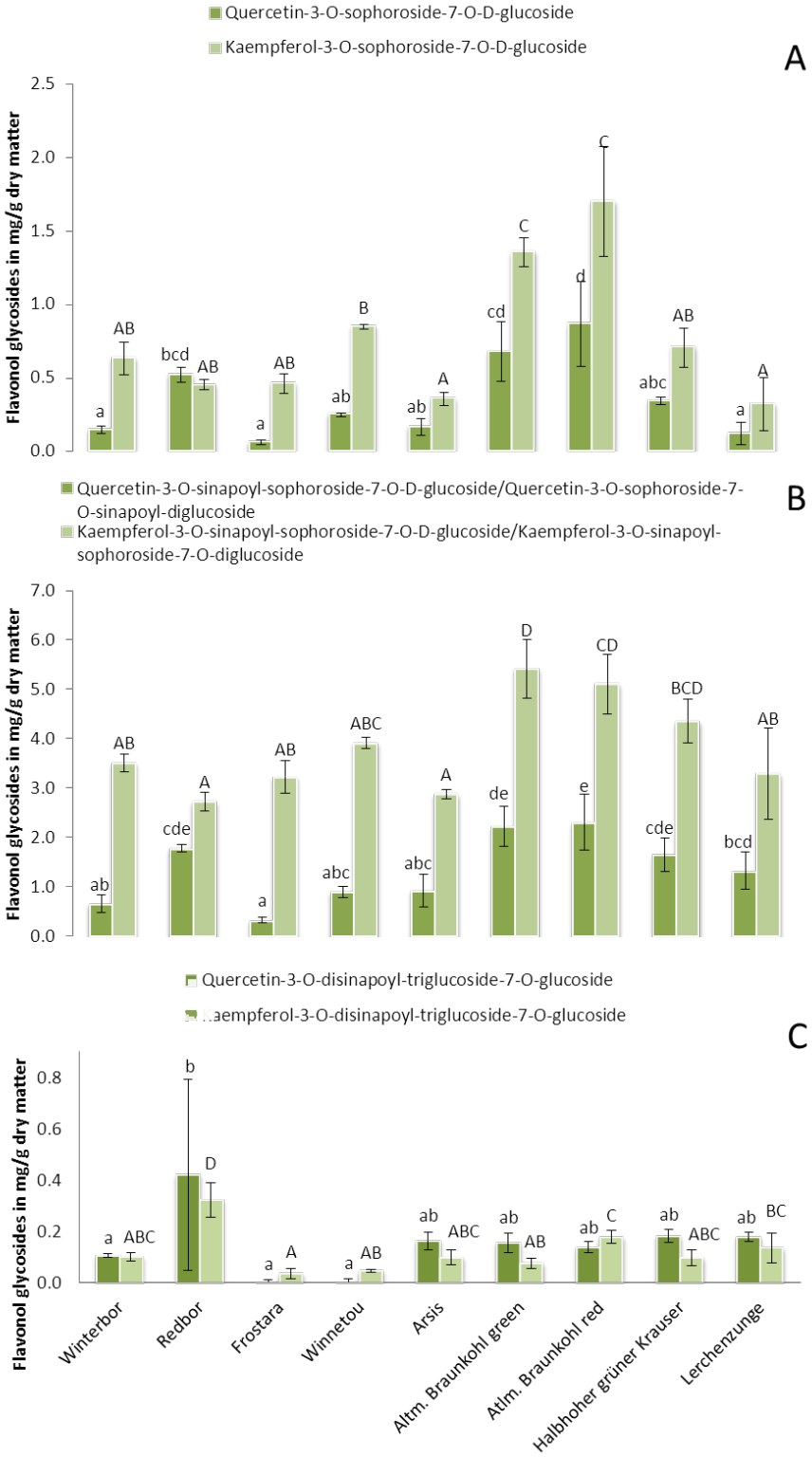

Figure 7.10: Structurally different flavonoid glycoside concentrations in kale cv. 'Winterbor'. A: non-acylated triglycosides; B: monoacylated triglycosides; C: diacylated tetraglycosides. The plants were grown in the field. Different letters indicate significant differences between the cultivars for each flavonol glycoside ($p \leq 0.05$ by Tukey's HSD test). Each value represents the mean of three replicates ± SD. Susanne Neugart, unpublished results.

Identification of Hydroxycinnamic Acid Derivatives

The identification of phenolic compounds is one of the major challenges in analytics today as there is a number of compounds and species differ enormously in their profiles and concentrations. The following is an example on the hydroxycinnamic acid derivatives in kale to highlight the process of the identification of phenolic compounds by (1) do a general literature research and find general fragmentation pattern for phenolics, (2) interpreting the fragmentation pattern measured and (3) compare to the literature if these compounds have been described before for the same genotype or species. From studies with HPLC-DAD-ESI-MSn in auto-mode to MS3, 30 hydroxycinnamic acid derivatives and a hydroxybenzoic acid glycoside (diprotuchatechuic acid-gentiobioside) were tentatively identified in kale (Fig. 7.11 and Table 7.1). The hydroxycinnamic acid derivatives can be classified into four aglycones, four quinic acid esters, four monoacylated hydroxycinnamic acid glycosides (mono-, di- and triglycosides), 12 diacylated hydroxycinnamic acid glycosides (di- and triglycoside) and six triacylated hydroxycinnamic acid glycosides (di- and triglycosides). For all identified glycosides, glucose was exclusively glycosylated – a finding that is also confirmed in the literature for other *Brassica* species (Ferreres, Fernandes, Sousa, et al. 2009; Harbaum et al. 2007; H. Olsen et al. 2009). In addition to the cleavage of 324 Da for diglucosides, the glycosides did not show the cleavage of 180 Da (characteristic of sophoroses). Thus, the diglucosides can be identified as gentiobioses, which is the case for kale (Ferreres, Fernandes, Sousa, et al. 2009; Lin and Harnly 2009).

As an example, the identification of hydroxycinnamic acid derivatives' based on fragmentation patterns is described. In the case of the triacylated hydroxycinnamic acid glycosides, three substances have the same fragmentation pattern. Starting from the deprotonated molecular ions m/z 929 (H30), m/z 945 (H25) and m/z 959 (H29), a sinapic acid (224 Da) was first cleaved in MS2 followed by the loss of a second sinapic acid or a sinapic acid residue. MS3 is characterized by the fragment ions [MH-224-224]- and [MH-224-206]-. In addition, the loss of a ferulic acid by the fragmentation [M-H-224-176]- was observed in the MS3 of substance H30. For all substances, MS3 showed the deprotonated molecular ions of hyroxyferulic acid (m/z 209) or sinapic acid (m/z 223). The substances were identified as disinapoyl-feruloyl gentiobioside (H30), disinapoyl-hydroxyferuloyl gentiobioside (H25) and trisinapoyl gentiobioside (H29). The substances disinapoyl-feruloyl gentiobioside (H30) and trisinapoyl gentiobioside (H29) have already been found in various *Brassica oleracea* (Ferreres, Fernandes, Oliveira, et al. 2009; Lin and Harnly 2009; H. Olsen et al. 2009, 2010) and *Brassica rapa* (Harbaum et al. 2007). The disinapoyl-hydroxyferuloyl gentiobioside (H25) has hitherto only been found in tronchuda cabbages (Ferreres, Fernandes, Oliveira, et al. 2009). A further substance shows in the MS the deprotonated molecular weight m/z 899. In MS2, the loss of a ferulic acid is characterized by the fragment ion [M-H-194]-. The MS3 shows both the cleavage of a sinapic acid residue ([M-H-194-206]-) as well as the cleavage of a ferulic acid ([M-H-194-194]-) and a ferulic acid residue. The substance is identified as sinapoyl-diferuloyl gentiobiose and has already been identified by (Ferreres, Fernandes, Sousa, et al. 2009) in tronchuda cabbage. For the exact identification measurements on high resolution mass spectrometry followed by NMR measurements would be necessary.

Method Validation

Which extraction method or measuring method is used depends on the scientific

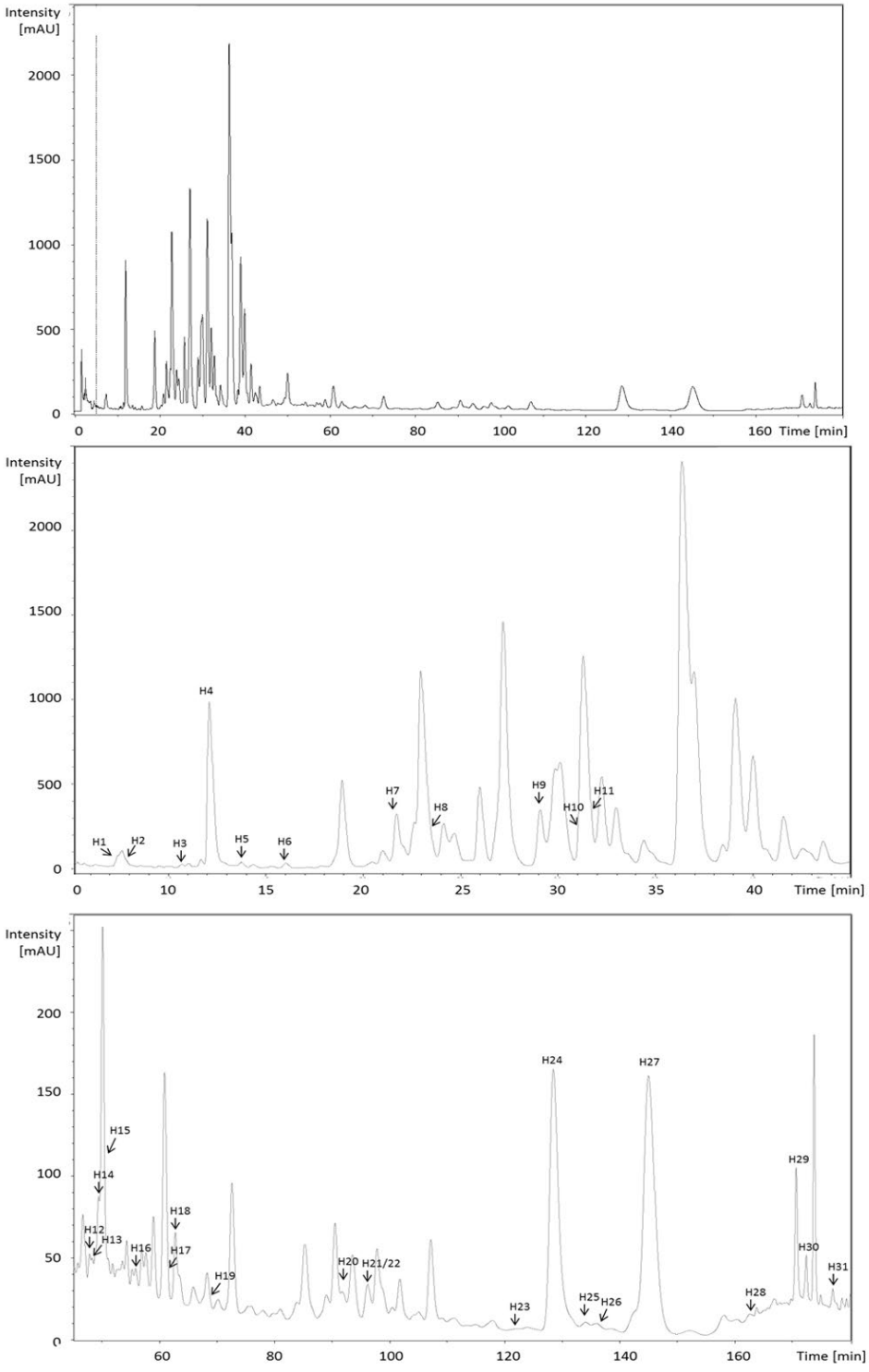

Figure 7.11: Chromatogram of flavonol glycosides and hydroxycinnamic acid derivatives of kale cv. 'Winterbor' at λ = 320 nm H1-H31 represent the tentatively identified hydroxycinnamic acid derivatives and a hydroxybenzoic acid glycoside (diprotuchatechuic acid-gentiobioside). The two lower panels are enlarged views for the initial 45 min of the run, and remaining of the run, respectively. Scale limits differ among panels. See Table 7.1 for additional details. Susanne Neugart, unpublished data.

Table 7.1: Fragmentation patterns of 30 hydroxycinnamic acid derivatives and one hydroxybenzoic acid derivative in kale. Susanne Neugart, unpublished data

	Tentative name	MS	MS2	MS3	Source
H01	feruloyl diglucoside	517	353		—
H02	diprotucatechuic acid gentiobiose	631	315	153	—
H03	feruloyl triglucoside	517, 677	179, 353, 341		—
H04	chlorogenic acid derivative	353	191		Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H05	caffeic acid glucoside	341	179		Harbaum et al. 2007
H06	hydroxyferulic acid glucoside	371	209		—
H07	chlorogenic acid derivative	353	191		Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H08	caffeic acid	179			Lin and Harnly 2009
H09	hydroxyferulic acid	209			Lin and Harnly 2009
H10	sinapic acid glucoside	385	223		Ferreres, Fernandes, Sousa, et al. 2009
H11	chlorogenic acid derivative	353	191		Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H12	ferulic acid	193			Lin and Harnly 2009
H13	disinapoyl gentiobioside	753	529	223	
H14	feruloyl quinic acid	367	191		Lin and Harnly 2009
H15	sinapic acid	223			Lin and Harnly 2009
H16	sinapoyl-caffeoyl triglucoside	871	709	485	—
H17	sinapoyl-coumaroyl triglucoside	855	693	469	—
H18	sinapoyl-feruloyl triglucoside	885	723	499	H. Olsen et al. 2009
H19	diferuloyl triglucoside	855	693	499	H. Olsen et al. 2009
H20	sinapoyl-hydroxyferuloyl gentiobioside	739	515	191	—
H21	disinapoyl-feruloyl triglucoside	1091	929	705	H. Olsen et al. 2009
H22	sinapoyl-caffeoyl gentiobioside	709	485	161	—
H23	trisinapoyl triglucoside	1121	959	735	H. Olsen et al. 2009
H24	disinapoyl gentiobioside	753	529	223	Ferreres, Fernandes, Sousa, et al. 2009; Harbaum et al. 2007; Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H25	disinapoyl-hydroxyferuloyl gentiobioside	945	721	515	—
H26	sinapoyl-coumaroyl gentiobioside	693	469	163	—
H27	sinapoyl-feruloyl gentiobioside	723	499	193	Ferreres, Fernandes, Sousa, et al. 2009; Harbaum et al. 2007; Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H28	diferuloyl-gentiobiose	693	499	193	Ferreres, Fernandes, Sousa, et al. 2009; Lin and Harnly 2009
H29	trisinapoyl gentiobioside	959	735	529	Ferreres, Fernandes, Sousa, et al. 2009; Harbaum et al. 2007; Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H30	disinapoyl-feruloyl gentiobioside	929	705	481, 499, 529	Ferreres, Fernandes, Sousa, et al. 2009; Harbaum et al. 2007; Lin and Harnly 2009; H. Olsen et al. 2009, 2010
H31	sinapoyl-diferuloyl gentiobiose	899	705	499	Ferreres, Fernandes, Sousa, et al. 2009

question that should be answered. Nevertheless, a method validation is the basis of reliable results. This includes optimization of the extraction, selectivity, peak purity, reproducibility (precision and accuracy), recovery, detection limit and quantification limit (if applicable), as well as calibration curve (linearity) (Bayram et al. 2013; Fucina et al. 2012; Gouveia and Castilho 2012; Li et al. 2012; Schoedl et al. 2011). If the quantification of compounds is performed with a mass spectrometer, other factors, such as ionization stability and ion suppression by the matrix, should also be considered. Importantly, a method validation should be conducted at the beginning of the establishment of an extraction or measuring method. To ensure that the established method is still correct, a double or triple determination of each sample is recommended and one or two reference standards should be measured with each sequence. Most of the parameters can then be rated in comparison with the validation conducted at the beginning. A new validation is necessary when something changed in the method, e.g. lower amounts of solutions during extraction, a new column of the same or other packing material, changes in the gradient or changes in the ionization process in the mass spectrometer.

The example shown here is the method validation performed for the flavonol aglycones in kale in which the compounds were quantified by HPLC.

Optimization of the Extraction

For the optimization of the extraction there are several factors to consider dependent on the extraction method (for different possible extraction methods please see Julkunen-Tiitto et al. 2015): extraction solution (including mixtures of polar and non-polar solution), extraction time and number of extractions, extraction temperature, sample weight, volumes of the extraction solution, shaking or mixing of the sample (Table 7.2). Exemplarily for kale the concentrations of HCl and methanol were not changed for the optimization of the kale extracts as these were established during previous experiments for broccoli (Krumbein et al. 2007). The investigation of the hydrolysis time (1, 2, 3, 4 and 6 h) on the flavonol aglycones quercetin, kaempferol and isorhamnetin occurring in kale showed that 2 hours were sufficient for acid hydrolysis to take place (50% methanol with 1.6 M HCl at 100°C).

Selectivity

The selectivity of the method is the sum of HPLC parameters that are optimized for the measurement of the phenolic compounds including choice of eluents, gradient, oven temperature, and detection wavelength. Therefore (1) sample extracts of kale (Fig. 7.8) and (2) standard mixtures of the available flavonoid aglycone standards (Fig. 7.9) were used to validate the separation of peaks and detection wavelength (chosen based on compounds' absorption spectrum) and the possible partial overlap of peaks due to elution times. This led to the selection of the eluents solvent A (99.5% water and 0.5% acetic acid) and solvent B (100% acetonitrile). The gradient and oven temperature were optimized. The detection wavelength for quercetin, kaempferol and isorhamnetin was chosen nearest to their measured absorption maxima and set at $\lambda = 370$ nm. For the method details, see section Flavonol Aglycones on page 41.

Peak Purity

The peak purity was verified by DAD (λ = 190-600 nm) by comparing peak shapes and the absorption spectrum at the key locations of a peak (base, before and after the peak; turning point, in the increasing and decreasing slope; and apex). For the relevant peaks of quercetin, kaempferol and isorhamnetin, no impurities or co-elutions were detected in

Table 7.2: Parameters of an extraction and their optimization.

Parameters of extraction	Optimization
solvent/extractant[a]	highly depends on the polarity of the target compounds, generally 50-70% methanol or ethanol are sufficient for the extraction of a wide number of phenolic compounds
length of time and number of extractions	the extraction time should be as long as necessary but as short as possible to avoid oxidation processes, the number of extractions for one extract can vary from 1 to 5
temperature	glycosides are more sensitive to temperature and might be degraded to aglycones above 40°C
sample mass	the more sample weight (5–500 mg) is used the lower is the variation coefficient of the reproducibility
volume of solvent/extractant	should be in relation to the sample weight, but the higher the volume the less concentration of phenolic compounds is found per ml so a concentration step for the extracts may be useful
shaking or mixing	for the better extraction shaking or mixing is essential during the whole extraction process

[a] Including mixtures of polar and non-polar solvents

kale, the species used here as example.

Reproducibility

To determine reproducibility, the precision (variation dependent on the HPLC measurement procedure by itself) as well as the accuracy (variation dependent on HPLC measurement procedure plus sample preparation, after acid hydrolysis) for the flavonol aglycones quercetin, kaempferol and isorhamnetin were investigated for kale. To determine precision, the *same* hydrolysed extract was injected 10 times, and average, standard deviation, as well as variation coefficient were calculated. Such calculations should be performed intra-day (within one day) and inter-day (over several days). To determine accuracy, 10 times 0.5 g of the freeze-dried kale was hydrolysed with 50% aqueous methanol and 1.6 M HCl as previously described (see section Flavonol Aglycones on page 41). The variation coefficient of accuracy was 3% for quercetin and kaempferol and 10% for isorhamnetin of which the variation coefficient of precision was below 1 % for all.

Stability

The stability of solutions is of special interest for polyhydroxylated flavonoids. Especially aglycones are degraded quickly both as standards as well as in the samples. For the standard stability, stock solutions of quercetin, kaempferol and isorhamnetin were prepared in the concentrations 1 mg/100 ml each. Aliquots of these were stored at 4°C and measured each day up to five days. The stability after one day was 96% for quercetin, 99% for kaempferol and 95% for isorhamnetin (Fig. 7.12). After five days, the stability was 52% for quercetin, 96% for kaempferol and 53% for isorhamnetin. The comparable results were observed for the samples of kale after acid hydrolysis. These highly differing percentages highlight that samples should be

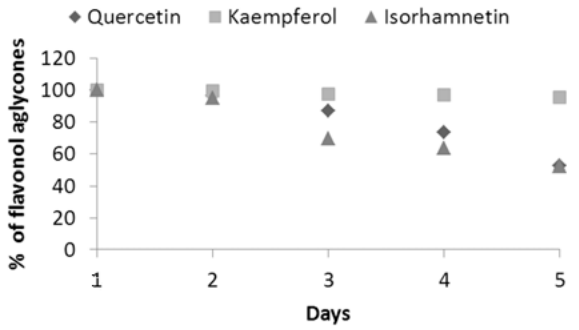

Figure 7.12: Stability of quercetin, kaempferol and isorhamnetin aglycones in kale extracts after acid hydrolysis.

prepared freshly and measured within one day after acid hydrolysis.

Flavonoid glycoside and hydrocycinnamic acid derivative standards (quercetin-3-O-glucoside, kaempferol-3-O-glucosides and isorhamnetin-3-O-glucosides, 3-chlorogenic acid) were stable (≥ 95%) under the same conditions for up to three months. Such stability was also observed for kale samples. Thus, these samples can be stored for weeks at 4°C.

Recovery

The recovery serves the review of the method concerning the quantitative evaluation. The aim is to increase the areas of the sample's flavonoids by 50% by addition of an appropriate standard. The recovery rate describes how much of the known added amount of standard contained in the measured sample is detected by the measuring procedure after extraction. To determine the recovery rate of the aglycones quercetin, kaempferol and isorhamnetin, three samples each of 0.5 g of freeze-dried kale alone, standards alone (0.5 ml each of quercetin and kaempferol (concentration 1 mg/10 ml) as well as 2 ml of isorhamnetin (concentration 15 mg/15 ml) and 0.5 g of freeze-dried kale plus the stated amounts of standards were hydrolysed with 50% aqueous methanol and 1.6 M HCl as previously described (see section Flavonol Aglycones on page 41). The aglycones were determined quantitatively using the HPLC-DAD method as previously described (see section Flavonol Aglycones on page 41). The recovery rate is defined as the ratio of the area of kale sample plus standards (x%) to the sum of the areas from the kale sample alone and the standards alone (100%). The recovery rate for quercetin (108%), kaempferol (112%) and isorhamnetin (110%) were higher than 100% which would lead to an overestimation of the results. Higher or lower recovery rates are a result of interactions of the standard compound with the matrix e.g. due to antioxidants in the matrix that stabilizes the standards. These recovery rates need to be included in the quantification to avoid over- or underestimation of compounds.

Detection Limit and Quantification Limit

The detection limit is the lowest concentration of detection of a target compound and was determined for the aglycone isorhamnetin as it occurs at low concentrations in kale. For this purpose, the signal-to-noise ratio was used to estimate the detection limit. The detection limit is reached when the noise (baseline) detected is exceed by a signal (peak) by a factor of 3. After acid hydrolysis, the kale sample was diluted and measured. The dilution for measuring the smallest signal was 1:20 and the dilution for measuring the noise was 1:100 (several others are measured). The detection limit was 276 ng/g dry matter with a peak at the dilution 1:20 that was approximately 3 times higher than the baseline of the dilution 1:100. The quantification limit is calculated based on the detection limit and should exceed the detection limit by 3 times. Thus, for isorhamnetin, the quantification limit is 828 ng/g dry matter.

Calibration Curves (linearity)

After the method validation the calibration curves for the quantification of compounds

are measured. Therefore, a range of minimum 4 known concentrations of reference standards are measured and the areas are used to generate a function. The most precise way is to use isotopic labeled standards. For quercetin, kaempferol and isorhamnetin for flavonoid analysis in kale the calibration curves were prepared with available reference standards. For the quantification of the aglycones quercetin, kaempferol and isorhamnetin standards (solved in 50% aqueous methanol) were measured externally. The concentrations were adjusted using pre-experiments with kale and measured in a range of 0.01–10 mg/100 ml. For quercetin (302.2 g/mol), the standard was quercetin dihydrate (338.3 g/mol). For the initial weight of 1 mg of quercetin to 10 ml, 1.12 mg of quercetin dihydrate was used. The equation of the reference standards were used for the calculation of the flavonoid concentration in kale.

Conclusion

The accurate analysis of phenolic compound profiles and concentrations in plants has been of interest for many years. However, to date, a standard procedure does not exist.

This article highlights the effect of abiotic factors on flavonoids and recommends that these should be considered while planning and conducting experiments. It is of high importance to equalize the growth conditions for plants under different treatments with only the factor(s) under study varying systematically. Proper randomization and precisely monitoring the experimental conditions helps ensure reproducibility of studies. We here have taken advantage that we have conducted a number of experiments on kale cv. 'Winterbor' covering responses to various abiotic factors. Using data from a single cultivar makes it easier to demonstrate the range and complexity of the responses. In kale, indoor experiments in climate chambers resulted in concentrations of kaemp-

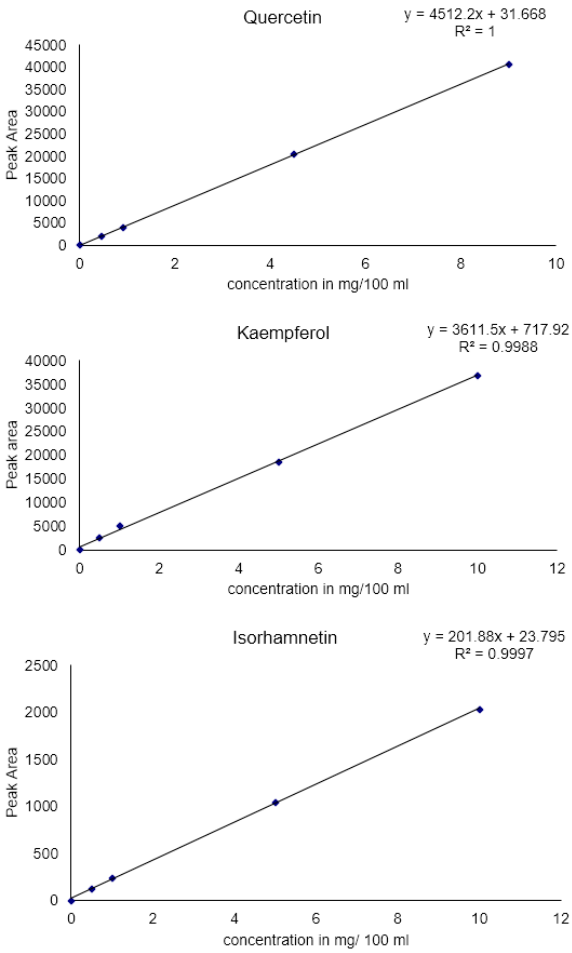

Figure 7.13: Calibration curves for the three aglycones quercetin, kaempferol and isorhamnetin. The integrated area under the peaks of the absorbance vs. time curves, plotted against the known concentration of the standard samples that were injected. The fitted equations, shown in the top right corner of each panel, are later used to convert peak areas from samples of unknown concentration into actual concentrations in the extracts.

ferol, the main aglycone of kale, ranging from 0.7 to 4.0 mg/g dry matter while quercetin varied from 0.6 to 2.1 mg/g dry matter dependent on the factor that was investigated. In the outdoor experiment on nitrogen fertilization the concentrations were much higher at 5.0–6.8 mg/g dry matter for kaempferol and 1.0–6.5 mg/g dry matter for quercetin. Of note is that the quercetin to kaempferol ratio changed dramatically. The observed pattern was that the quercetin to kaempferol ratio increased with increasing plant age, in young leaves, with low nitrogen supply, with high irradiance (photosynthetically active and UVB radiation). A ranking of which of these factors has a stronger impact is difficult, if not impossible, to establish as this requires setting a "reference" condition for each factor. Interactions among factors can be expected as well. In conclusion, in studies of mechanisms, controlled environment experiments should be favored to exclude uncontrolled variation in biotic and abiotic factors. Field experiments provide more realistic conditions, but are subject to temporal and spatial variation. This means that continuous monitoring of environmental conditions must be routinely done and the resulting data reported. For example, daily irradiance during growth up to the harvest needs to be reported as plants accumulate phenolic compounds dependent on the accumulated exposure to radiation (Del-Castillo-Alonso et al. 2016). Furthermore concentrations at the time of metabolite measurement, also depend on sample storage: i.e. low humidity of the samples is more important than the temperature during storage in the case of phenolic metabolites.

Several different methods can be used to measure profiles and concentrations of phenolic compounds. The decision of which method to use should be related to the scientific question. The total phenolic content is a fast and cheap method to gain preliminary information about the extracts. However, specific identification and quantification of phenolic compounds is not obtained. A more detailed analysis of flavonoid aglycones and aglycones of other phenolic compounds is useful for a number of questions related to the beneficial effects of plant phenolic compounds in humans and how large an effect can be expected. However, the most suitable method to answer questions related to metabolism and function in plants is the analysis of flavonoid glycosides and glycosides of other phenolic compounds. This method is time-consuming, expensive and needs good analytical skills to be able to achieve a correct identification and quantification based on HPLC and mass spectrometric data. Nevertheless, one should be aware that structurally different phenolic compounds might respond differently to biotic and abiotic factors. It is not possible to rank the methods as all of them are useful for different research questions, consequently the question under study should drive the selection of the analytical method.

Nevertheless, method validation is the basis of reliable results and should be performed in advance of the measurement of samples from experiments so as to establish the quality of the data to be acquired. A new validation is required whenever the plant species, the extraction method and/or any other significant aspect of the protocol are changed. As validation is the basis of reliable and consistent results, one should take the time and do a proper valid as frequently as needed. One of the most important aspect is reproducibility, which can be monitored by means of double or triple parallel determinations of each sample or by including one or two reference standards in each batch of samples measured in the laboratory.

Acknowledgements I sincerely thank the DFG (Deutsche Forschungsgemeinschaft) for funding (projects: KR-2066/3-1 and KR-1452/12-1) and the co-authors: Michaela Fiol, Angelika Krumbein, Lothar W. Kroh, Sascha Rohn, Rita Zrenner, Hans-Peter Kläring,

Susann Adermann, Annika Weckmüller and co-workers: Andrea Jankowsky, Annett Platalla, Andrea Maikath, Elke Büsch, Ursula Zentner, both form the Leibniz-Institute of Vegetable and Ornamental Crops and TU Berlin.

References

Bayram, B., B. Ozcelik, G. Schultheiss, J. Frank, and G. Rimbach (2013). "A validated method for the determination of selected phenolics in olive oil using high-performance liquid chromatography with coulometric electrochemical detection and a fused-core column". In: *Food Chemistry* 138.2-3, pp. 1663–1669. DOI: 10.1016/j.foodchem.2012.11.122.

Becker, C., H.-P. Kläring, L. W. Kroh, and A. Krumbein (2013). "Temporary reduction of radiation does not permanently reduce flavonoid glycosides and phenolic acids in red lettuce". In: *Plant Physiology and Biochemistry* 72, pp. 154–160. DOI: 10.1016/j.plaphy.2013.05.006.

Bernal, M., L. Llorens, R. Julkunen-Tiitto, J. Badosa, and D. Verdaguer (2013). "Altitudinal and seasonal changes of phenolic compounds in Buxus sempervirens leaves and cuticles". In: *Plant Physiology and Biochemistry* 70, pp. 471–482. DOI: 10.1016/j.plaphy.2013.06.012.

Bernal, M., D. Verdaguer, J. Badosa, A. Abadía, J. Llusià, J. Peñuelas, E. Núñez-Olivera, and L. Llorens (2015). "Effects of enhanced UV radiation and water availability on performance, biomass production and photoprotective mechanisms of *Laurus nobilis* seedlings". In: *Environmental and Experimental Botany* 109, pp. 264–275. DOI: 10.1016/j.envexpbot.2014.06.016.

Bilger, W., M. Rolland, and L. Nybakken (2007). "UV screening in higher plants induced by low temperature in the absence of UV-B radiation". In: *Photochemical & Photobiological Sciences* 6.2, p. 190. DOI: 10.1039/b609820g.

Buendía, B., M. I. Gil, J. A. Tudela, A. L. Gady, J. J. Medina, C. Soria, J. M. López, and F. A. Tomás-Barberán (2010). "HPLC-MS Analysis of Proanthocyanidin Oligomers and Other Phenolics in 15 Strawberry Cultivars". In: *Journal of Agricultural and Food Chemistry* 58.7, pp. 3916–3926. DOI: 10.1021/jf9030597.

Calderon-Montano, J. M., E. Burgos-Moron, C. Perez-Guerrero, and M. Lopez-Lazaro (2011). "A Review on the Dietary Flavonoid Kaempferol". In: *Mini-Reviews in Medicinal Chemistry* 11.4, pp. 298–344. DOI: 10.2174/138955711795305335.

Castillo-Muñoz, N., S. Gómez-Alonso, E. García-Romero, and I. Hermosín-Gutiérrez (2010). "Flavonol profiles of *Vitis vinifera* white grape cultivars". In: *Journal of Food Composition and Analysis* 23.7, pp. 699–705. DOI: 10.1016/j.jfca.2010.03.017.

Chennupati, P., P. Seguin, R. Chamoun, and S. Jabaji (2012). "Effects of High-Temperature Stress on Soybean Isoflavone Concentration and Expression of Key Genes Involved in Isoflavone Synthesis". In: *Journal of Agricultural and Food Chemistry* 60.51, pp. 12421–12427. DOI: 10.1021/jf3036319.

Dall'Acqua, S., G. Miolo, G. Innocenti, and S. Caffieri (2012). "The Photodegradation of Quercetin: Relation to Oxidation". In: *Molecules* 17.12, pp. 8898–8907. DOI: 10.3390/molecules17088898.

De Pascale, S., A. Maggio, R. Pernice, V. Fogliano, and G. Barbieri (2007). "Sulphur fertilization may improve the nutritional value of *Brassica rapa* L. subsp. *sylvestris*". In: *European Journal of Agronomy* 26.4, pp. 418–424. DOI: 10.1016/j.eja.2006.12.009.

Del-Castillo-Alonso, M. Á., A. Castagna, K. Csepregi, É. Hideg, G. Jakab, M. A. K. Jansen, T. Jug, L. Llorens, A. Mátai, J. Martínez-Lüscher, et al. (2016). "Environmental Factors Correlated with the Metabolite Profile of *Vitis vinifera* cv. Pinot Noir Berry Skins along a European Latitudinal

Gradient". In: *Journal of Agricultural and Food Chemistry* 64.46, pp. 8722–8734. DOI: 10.1021/acs.jafc.6b03272.

Demotes-Mainard, S., T. Péron, A. Corot, J. Bertheloot, J. L. Gourrierec, S. Pelleschi-Travier, L. Crespel, P. Morel, L. Huché-Thélier, R. Boumaza, et al. (2016). "Plant responses to red and far-red lights, applications in horticulture". In: *Environmental and Experimental Botany* 121, pp. 4–21. DOI: 10.1016/j.envexpbot.2015.05.010.

Edwards, R., S. A. Tiller, and A. D. Parry (1997). "The effect of plant age and nodulation on the isoflavonoid content of red clover (*Trifolium pratense*)". In: *Journal of Plant Physiology* 150.5, pp. 603–610. DOI: 10.1016/s0176-1617(97)80326-4.

El Morchid, E. M., P. T. Londoño, M. Papagiannopoulos, L. Gobbo-Neto, and C. Müller (2014). "Variation in flavonoid pattern in leaves and flowers of *Primula veris* of different origin and impact of UV-B". In: *Biochemical Systematics and Ecology* 53, pp. 81–88. DOI: 10.1016/j.bse.2013.12.032.

Fallovo, C., M. Schreiner, D. Schwarz, G. Colla, and A. Krumbein (2011). "Phytochemical Changes Induced by Different Nitrogen Supply Forms and Radiation Levels in Two Leafy *Brassica* Species". In: *Journal of Agricultural and Food Chemistry* 59.8, pp. 4198–4207. DOI: 10.1021/jf1048904.

Ferreres, F., F. Fernandes, J. M. Oliveira, P. Valentão, J. A. Pereira, and P. B. Andrade (2009). "Metabolic profiling and biological capacity of Pieris brassicae fed with kale (*Brassica oleracea* L. var. *acephala*)". In: *Food and Chemical Toxicology* 47.6, pp. 1209–1220. DOI: 10.1016/j.fct.2009.02.014.

Ferreres, F., F. Fernandes, C. Sousa, P. Valentão, J. A. Pereira, and P. B. Andrade (2009). "Metabolic and bioactivity insights into *Brassica oleracea* var. *acephala*". In: *Journal of Agricultural and Food Chemistry* 57.19, pp. 8884–8892.

Fiol, M., S. Adermann, S. Neugart, S. Rohn, C. Mügge, M. Schreiner, A. Krumbein, and L. W. Kroh (2012). "Highly glycosylated and acylated flavonols isolated from kale (*Brassica oleracea* var. *sabellica*) — Structure–antioxidant activity relationship". In: *Food Research International* 47.1, pp. 80–89. DOI: 10.1016/j.foodres.2012.01.014.

Flanigan, P. M. and E. D. Niemeyer (2014). "Effect of cultivar on phenolic levels, anthocyanin composition, and antioxidant properties in purple basil (*Ocimum basilicum* L.)" In: *Food Chemistry* 164, pp. 518–526. DOI: 10.1016/j.foodchem.2014.05.061.

Fucina, G., L. C. Block, T. Baccarin, T. R. G. Ribeiro, N. L. M. Quintão, V. C. Filho, R. M. L. Silva, and T. M. B. Bresolin (2012). "Development and validation of a stability indicative HPLC–PDA method for kaurenoic acid in spray dried extracts of *Sphagneticola trilobata* (L.) Pruski, *Asteraceae*". In: *Talanta* 101, pp. 530–536. DOI: 10.1016/j.talanta.2012.09.024.

Gadkari, P. V. and M. Balaraman (2015). "Catechins: Sources, extraction and encapsulation: A review". In: *Food and Bioproducts Processing* 93, pp. 122–138. DOI: 10.1016/j.fbp.2013.12.004.

Gliszczyńska-Świgło, A., A. Kałużewicz, K. Lemańska, M. Knaflewski, and B. Tyrakowska (2007). "The effect of solar radiation on the flavonol content in broccoli inflorescence". In: *Food Chemistry* 100.1, pp. 241–245. DOI: 10.1016/j.foodchem.2005.09.048.

Gouveia, S. C. and P. C. Castilho (2012). "Validation of a HPLC-DAD–ESI/MSn method for caffeoylquinic acids separation, quantification and identification in medicinal *Helichrysum* species from Macaronesia". In: *Food Research International* 45.1, pp. 362–368. DOI: 10.1016/j.foodres.2011.09.023.

Groenbaek, M., S. Jensen, S. Neugart, M. Schreiner, U. Kidmose, and H. L. Kristensen (2016). "Nitrogen split dose fertilization,

plant age and frost effects on phytochemical content and sensory properties of curly kale (*Brassica oleracea* L. var. *sabellica*)". In: *Food Chemistry* 197, pp. 530–538. DOI: 10.1016/j.foodchem.2015.10.108.

Groenbaek, M., S. Jensen, S. Neugart, M. Schreiner, U. Kidmose, and H. L. Kristensen (2014). "Influence of Cultivar and Fertilizer Approach on Curly Kale (*Brassica oleracea* L. var. *sabellica*). 1. Genetic Diversity Reflected in Agronomic Characteristics and Phytochemical Concentration". In: *Journal of Agricultural and Food Chemistry* 62.47, pp. 11393–11402. DOI: 10.1021/jf503096p.

Guidi, L., C. Brunetti, A. Fini, G. Agati, F. Ferrini, A. Gori, and M. Tattini (2016). "UV radiation promotes flavonoid biosynthesis, while negatively affecting the biosynthesis and the de-epoxidation of xanthophylls: Consequence for photoprotection?" In: *Environmental and Experimental Botany* 127, pp. 14–25. DOI: 10.1016/j.envexpbot.2016.03.002.

Hagen, S. F., G. I. A. Borge, K. A. Solhaug, and G. B. Bengtsson (2009). "Effect of cold storage and harvest date on bioactive compounds in curly kale (*Brassica oleracea* L. var. *acephala*)". In: *Postharvest Biology and Technology* 51.1, pp. 36–42. DOI: 10.1016/j.postharvbio.2008.04.001.

Häkkinen, S. H. and A. Törrönen (2000). "Content of flavonols and selected phenolic acids in strawberries and Vaccinium species: influence of cultivar, cultivation site and technique". In: *Food Research International* 33.6, pp. 517–524. DOI: 10.1016/s0963-9969(00)00086-7.

Halac, S., V. Villafañe, R. Gonçalves, and E. Helbling (2014). "Photochemical responses of three marine phytoplankton species exposed to ultraviolet radiation and increased temperature: Role of photoprotective mechanisms". In: *Journal of Photochemistry and Photobiology B: Biology* 141, pp. 217–227. DOI: 10.1016/j.jphotobiol.2014.09.022.

Han, Y., S. Vimolmangkang, R. E. Soria-Guerra, S. Rosales-Mendoza, D. Zheng, A. V. Lygin, and S. S. Korban (2010). "Ectopic Expression of Apple F3'H Genes Contributes to Anthocyanin Accumulation in the *Arabidopsis tt7* Mutant Grown Under Nitrogen Stress". In: *PLANT PHYSIOLOGY* 153.2, pp. 806–820. DOI: 10.1104/pp.109.152801.

Harbaum, B., E. M. Hubbermann, C. Wolff, R. Herges, Z. Zhu, and K. Schwarz (2007). "Identification of Flavonoids and Hydroxycinnamic Acids in Pak Choi Varieties (*Brassica campestris* L. ssp. *chinensis* var. *communis*) by HPLC–ESI-MS n and NMR and Their Quantification by HPLC–DAD". In: *Journal of Agricultural and Food Chemistry* 55.20, pp. 8251–8260. DOI: 10.1021/jf071314+.

Heimler, D., P. Vignolini, M. G. Dini, F. F. Vincieri, and A. Romani (2006). "Antiradical activity and polyphenol composition of local Brassicaceae edible varieties". In: *Food Chemistry* 99.3, pp. 464–469. DOI: 10.1016/j.foodchem.2005.07.057.

Hertog, M. G. L., P. C. H. Hollman, and M. B. Katan (1992). "Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands". In: *Journal of Agricultural and Food Chemistry* 40.12, pp. 2379–2383. DOI: 10.1021/jf00024a011.

Huang, J., M. Gu, Z. Lai, B. Fan, K. Shi, Y.-H. Zhou, J.-Q. Yu, and Z. Chen (2010). "Functional Analysis of the Arabidopsis PAL Gene Family in Plant Growth, Development, and Response to Environmental Stress". In: *Plant Physiology* 153.4, pp. 1526–1538. DOI: 10.1104/pp.110.157370.

Huang, Z., B. Wang, D. H. Eaves, J. M. Shikany, and R. D. Pace (2007). "Phenolic compound profile of selected vegetables frequently consumed by African Americans in the southeast United States". In: *Food Chemistry* 103.4, pp. 1395–1402. DOI: 10.1016/j.foodchem.2006.10.077.

Huché-Thélier, L., L. Crespel, J. L. Gourrierec, P. Morel, S. Sakr, and N. Leduc (2016). "Light signaling and plant responses to blue and UV radiations—Perspectives for applications in horticulture". In: *Environmental and Experimental Botany* 121, pp. 22–38. DOI: 10.1016/j.envexpbot.2015.06.009.

Jaakola, L., K. Määttä-Riihinen, S. Kärenlampi, and A. Hohtola (2004). "Activation of flavonoid biosynthesis by solar radiation in bilberry (Vaccinium myrtillus L) leaves." eng. In: *Planta* 218.5, pp. 721–728. DOI: 10.1007/s00425-003-1161-x.

Jansen, M. A. K. (2012). "Ultraviolet-B radiation: from stressor to regulatory signal." In: *Plant stress physiology*. Ed. by S. Shabala. CABI. Chap. 12, pp. 266–290. ISBN: 9781845939953. DOI: 10.1079/9781845939953.0266.

Johkan, M., K. Shoji, F. Goto, S.-N. Hashida, and T. Yoshihara (2010). "Blue Light-emitting Diode Light Irradiation of Seedlings Improves Seedling Quality and Growth after Transplanting in Red Leaf Lettuce". In: *HortScience* 45, pp. 1809–1814.

Julkunen-Tiitto, R., N. Nenadis, S. Neugart, M. Robson, G. Agati, J. Vepsäläinen, G. Zipoli, L. Nybakken, B. Winkler, and M. A. K. Jansen (2015). "Assessing the response of plant flavonoids to UV radiation: an overview of appropriate techniques". In: *Phytochemistry Reviews* 14.2, pp. 273–297. DOI: 10.1007/s11101-014-9362-4.

Klem, K., P. Holub, M. Štroch, J. Nezval, V. Špunda, J. Tříska, M. A. Jansen, T. M. Robson, and O. Urban (2015). "Ultraviolet and photosynthetically active radiation can both induce photoprotective capacity allowing barley to overcome high radiation stress". In: *Plant Physiology and Biochemistry* 93, pp. 74–83. DOI: 10.1016/j.plaphy.2015.01.001.

Klepacka, J., E. Gujska, and J. Michalak (2011). "Phenolic Compounds as Cultivar- and Variety-distinguishing Factors in Some Plant Products". In: *Plant Foods for Human Nutrition* 66.1, pp. 64–69. DOI: 10.1007/s11130-010-0205-1.

Kovinich, N., G. Kayanja, A. Chanoca, K. Riedl, M. S. Otegui, and E. Grotewold (2014). "Not all anthocyanins are born equal: distinct patterns induced by stress in *Arabidopsis*". In: *Planta* 240.5, pp. 931–940. DOI: 10.1007/s00425-014-2079-1.

Krumbein, A., H. Saeger-Fink, and I. Schonhof (2007). "Changes in quercetin and kaempferol concentrations during broccoli head ontogeny in three broccoli cultivars". In: *Journal of Applied Botany and Food Quality-Angewandte Botanik* 81, pp. 136–139.

Lavola, A., L. Nybakken, M. Rousi, J. Pusenius, M. Petrelius, S. Kellomäki, and R. Julkunen-Tiitto (2013). "Combination treatment of elevated UVB radiation, CO_2 and temperature has little effect on silver birch (*Betula pendula*) growth and phytochemistry". In: *Physiologia Plantarum* 149.4, pp. 499–514. DOI: 10.1111/ppl.12051.

Li, D., N. Martini, Z. Wu, and J. Wen (2012). "Development of an isocratic HPLC method for catechin quantification and its application to formulation studies". In: *Fitoterapia* 83.7, pp. 1267–1274. DOI: 10.1016/j.fitote.2012.06.006.

Lin, L.-Z. and J. M. Harnly (2009). "Identification of the Phenolic Components of Collard Greens, Kale, and Chinese Broccoli". In: *Journal of Agricultural and Food Chemistry* 57.16, pp. 7401–7408. DOI: 10.1021/jf901121v.

Løvdal, T., K. M. Olsen, R. Slimestad, M. Verheul, and C. Lillo (2010). "Synergetic effects of nitrogen depletion, temperature, and light on the content of phenolic compounds and gene expression in leaves of tomato". In: *Phytochemistry* 71.5-6, pp. 605–613. DOI: 10.1016/j.phytochem.2009.12.014.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951). "Protein Measurement with the Folin Phenol Reagent". In: *Journal of Biological Chemistry* 193.1, pp. 265–275.

Luis, J., R. M. Pérez, and F. V. González (2007). "UV-B radiation effects on foliar concentrations of rosmarinic and carnosic acids in rosemary plants". In: *Food Chemistry* 101.3, pp. 1211–1215. DOI: 10.1016/j.foodchem.2006.03.023.

Luo, C., X. Wang, G. Gao, L. Wang, Y. Li, and C. Sun (2013). "Identification and quantification of free, conjugate and total phenolic compounds in leaves of 20 sweetpotato cultivars by HPLC–DAD and HPLC–ESI–MS/MS". In: *Food Chemistry* 141.3, pp. 2697–2706. DOI: 10.1016/j.foodchem.2013.05.009.

Luthria, D. L., S. Mukhopadhyay, and D. T. Krizek (2006). "Content of total phenolics and phenolic acids in tomato (*Lycopersicon esculentum* Mill.) fruits as influenced by cultivar and solar UV radiation". In: *Journal of Food Composition and Analysis* 19.8, pp. 771–777. DOI: 10.1016/j.jfca.2006.04.005.

Lv, Y.-W., W. Hu, Y.-L. Wang, L.-F. Huang, Y.-B. He, and X.-Z. Xie (2011). "Identification and Determination of Flavonoids in *Astragali Radix* by High Performance Liquid Chromatography Coupled with DAD and ESI-MS Detection". In: *Molecules* 16.12, pp. 2293–2303. DOI: 10.3390/molecules16032293.

Lv, Z., X. Zhang, L. Liu, Y. Guo, Y. Fan, X. Yang, Y. Li, and W. Zhang (2013). "Comparing intraspecific responses of 12 winter wheat cultivars to different doses of ultraviolet-B radiation". In: *Journal of Photochemistry and Photobiology B: Biology* 119, pp. 1–8. DOI: 10.1016/j.jphotobiol.2012.12.002.

Martínez-Lüscher, J., F. Morales, S. Delrot, M. Sánchez-Díaz, E. Gomés, J. Aguirreolea, and I. Pascual (2013). "Short- and long-term physiological responses of grapevine leaves to UV-B radiation". In: *Plant Science* 213, pp. 114–122. DOI: 10.1016/j.plantsci.2013.08.010.

Merken, H. M., C. D. Merken, and G. R. Beecher (2001). "Kinetics Method for the Quantitation of Anthocyanidins, Flavonols, and Flavones in Foods". In: *Journal of Agricultural and Food Chemistry* 49.6, pp. 2727–2732. DOI: 10.1021/jf001266s.

Mølmann, J. A., A. L. Steindal, G. B. Bengtsson, R. Seljåsen, P. Lea, J. Skaret, and T. J. Johansen (2015). "Effects of temperature and photoperiod on sensory quality and contents of glucosinolates, flavonols and vitamin C in broccoli florets". In: *Food Chemistry* 172, pp. 47–55. DOI: 10.1016/j.foodchem.2014.09.015.

Morales, L. O., R. Tegelberg, M. Brosche, M. Keinanen, A. Lindfors, and P. J. Aphalo (2010). "Effects of solar UV-A and UV-B radiation on gene expression and phenolic accumulation in *Betula pendula* leaves". In: *Tree Physiology* 30.7, pp. 923–934. DOI: 10.1093/treephys/tpq051.

Mori, K., N. Goto-Yamamoto, M. Kitayama, and K. Hashizume (2007). "Effect of high temperature on anthocyanin composition and transcription of flavonoid hydroxylase genes in 'Pinot noir' grapes (*Vitis vinifera*)". In: *The Journal of Horticultural Science and Biotechnology* 82.2, pp. 199–206. DOI: 10.1080/14620316.2007.11512220.

Nascimento, L. B. S., M. V. Leal-Costa, M. A. S. Coutinho, N. dos S. Moreira, C. L. S. Lage, N. dos S. Barbi, S. S. Costa, and E. S. Tavares (2012). "Increased Antioxidant Activity and Changes in Phenolic Profile of *Kalanchoe pinnata* (Lamarck) Persoon (*Crassulaceae*) Specimens Grown Under Supplemental Blue Light". In: *Photochemistry and Photobiology* 89.2, pp. 391–399. DOI: 10.1111/php.12006.

Neugart, S., S. Baldermann, B. Ngwene, J. Wesonga, and M. Schreiner (2017). "Indigenous leafy vegetables of Eastern Africa — A source of extraordinary secondary plant metabolites". In: *Food Research International*. DOI: 10.1016/j.foodres.2017.02.014.

Neugart, S., M. Fiol, M. Schreiner, S. Rohn, R. Zrenner, L. W. Kroh, and A. Krumbein (2013). "Low and moderate photosynthetically active radiation affects the flavonol

glycosides and hydroxycinnamic acid derivatives in kale (*Brassica oleracea* var. *sabellica*) dependent on two low temperatures". In: *Plant Physiology and Biochemistry* 72, pp. 161–168. DOI: 10.1016/j.plaphy.2013.04.002.

– (2014). "Interaction of Moderate UV-B Exposure and Temperature on the Formation of Structurally Different Flavonol Glycosides and Hydroxycinnamic Acid Derivatives in Kale (*Brassica oleracea* var. *sabellica*)". In: *Journal of Agricultural and Food Chemistry* 62.18, pp. 4054–4062. DOI: 10.1021/jf4054066.

Neugart, S., H.-P. Kläring, M. Zietz, M. Schreiner, S. Rohn, L. W. Kroh, and A. Krumbein (2012). "The effect of temperature and radiation on flavonol aglycones and flavonol glycosides of kale (*Brassica oleracea* var. *sabellica*)". In: *Food Chemistry* 133.4, pp. 1456–1465. DOI: 10.1016/j.foodchem.2012.02.034.

Neugart, S., A. Krumbein, and R. Zrenner (2016). "Influence of Light and Temperature on Gene Expression Leading to Accumulation of Specific Flavonol Glycosides and Hydroxycinnamic Acid Derivatives in Kale (*Brassica oleracea* var. *sabellica*)". In: *Frontiers in Plant Science* 7. DOI: 10.3389/fpls.2016.00326.

Neugart, S., S. Rohn, and M. Schreiner (2015). "Identification of complex, naturally occurring flavonoid glycosides in Vicia faba and Pisum sativum leaves by HPLC-DAD-ESI-MSn and the genotypic effect on their flavonoid profile". In: *Food Research International* 76, pp. 114–121. DOI: 10.1016/j.foodres.2015.02.021.

Neugart, S., M. Zietz, M. Schreiner, S. Rohn, L. W. Kroh, and A. Krumbein (2012). "Structurally different flavonol glycosides and hydroxycinnamic acid derivatives respond differently to moderate UV-B radiation exposure". In: *Physiologia Plantarum* 145.4, pp. 582–593. DOI: 10.1111/j.1399-3054.2012.01567.x.

Nguyen, P. M. and E. D. Niemeyer (2008). "Effects of Nitrogen Fertilization on the Phenolic Composition and Antioxidant Properties of Basil (*Ocimum basilicum* L.)" In: *Journal of Agricultural and Food Chemistry* 56.18, pp. 8685–8691. DOI: 10.1021/jf801485u.

Olsen, H., K. Aaby, and G. I. A. Borge (2009). "Characterization and Quantification of Flavonoids and Hydroxycinnamic Acids in Curly Kale (*Brassica oleracea* L. convar. *acephala* var. *sabellica*) by HPLC-DAD-ESI-MSn". In: *Journal of Agricultural and Food Chemistry* 57.7, pp. 2816–2825. DOI: 10.1021/jf803693t.

– (2010). "Characterization, Quantification, and Yearly Variation of the Naturally Occurring Polyphenols in a Common Red Variety of Curly Kale (*Brassica oleracea* L. convar. *acephala* var. *sabellica* cv. 'Redbor')". In: *Journal of Agricultural and Food Chemistry* 58.21, pp. 11346–11354. DOI: 10.1021/jf102131g.

Olsen, K. M., R. Slimestad, U. S. Lea, C. Brede, T. LØvdal, P. Ruoff, M. Verheul, and C. Lillo (2009). "Temperature and nitrogen effects on regulators and products of the flavonoid pathway: experimental and kinetic model studies". In: *Plant, Cell & Environment* 32.3, pp. 286–299. DOI: 10.1111/j.1365-3040.2008.01920.x.

Pérez-Gregorio, R. M., M. S. García-Falcón, J. Simal-Gándara, A. S. Rodrigues, and D. P. F. Almeida (2010). "Identification and quantification of flavonoids in traditional cultivars of red and white onions at harvest". In: *Journal of Food Composition and Analysis* 23.6, pp. 592–598. DOI: 10.1016/j.jfca.2009.08.013.

Reifenrath, K. and C. Müller (2007). "Species-specific and leaf-age dependent effects of ultraviolet radiation on two *Brassicaceae*". In: *Phytochemistry* 68.6, pp. 875–885. DOI: 10.1016/j.phytochem.2006.12.008.

Ryan, K. G., K. R. Markham, S. J. Bloor, J. M. Bradley, K. A. Mitchell, and B. R. Jordan (1998). "UVB Radiation Induced Increase in

Quercetin:Kaempferol Ratio in Wild-Type and Transgenic Lines of *Petunia*". In: *Photochemistry and Photobiology* 68.3, pp. 323–330. DOI: 10.1111/j.1751-1097.1998.tb09689.x.

Schmidt, S., M. Zietz, M. Schreiner, S. Rohn, L. W. Kroh, and A. Krumbein (2010a). "Genotypic and climatic influences on the concentration and composition of flavonoids in kale (*Brassica oleracea* var. *sabellica*)". In: *Food Chemistry* 119.4, pp. 1293–1299. DOI: 10.1016/j.foodchem.2009.09.004.

– (2010b). "Identification of complex, naturally occurring flavonoid glycosides in kale (*Brassica oleracea* var. *sabellica*) by high-performance liquid chromatography diode-array detection/electrospray ionization multi-stage mass spectrometry". In: *Rapid Communications in Mass Spectrometry* 24.14, pp. 2009–2022. DOI: 10.1002/rcm.4605.

Schoedl, K., A. Forneck, M. Sulyok, and R. Schuhmacher (2011). "Optimization, In-House Validation, and Application of a Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS)-Based Method for the Quantification of Selected Polyphenolic Compounds in Leaves of Grapevine (*Vitis vinifera* L.)" In: *Journal of Agricultural and Food Chemistry* 59.20, pp. 10787–10794. DOI: 10.1021/jf202753g.

Schoedl, K., R. Schuhmacher, and A. Forneck (2012). "Studying the polyphenols of grapevine leaves according to age and insertion level under controlled conditions". In: *Scientia Horticulturae* 141, pp. 37–41. DOI: 10.1016/j.scienta.2012.04.014.

Siipola, S. M., T. Kotilainen, N. Sipari, L. O. Morales, A. V. Lindfors, T. M. Robson, and P. J. Aphalo (2014). "Epidermal UV-A absorbance and whole-leaf flavonoid composition in pea respond more to solar blue light than to solar UV radiation". In: *Plant, Cell & Environment* 38.5, pp. 941–952. DOI: 10.1111/pce.12403.

Stracke, R., J.-j. Favory, H. Gruber, L. Bartelniewoehner, S. Bartels, M. Binkert, M. Funk, B. Weisshaar, and R. Ulm (2010). "The *Arabidopsis* bZIP transcription factor HY5 regulates expression of the PFG1/MYB12 gene in response to light and ultraviolet-B radiation". In: *Plant, Cell & Environment*. DOI: 10.1111/j.1365-3040.2009.02061.x.

Strissel, T., H. Halbwirth, U. Hoyer, C. Zistler, K. Stich, and D. Treutter (2005). "Growth-Promoting Nitrogen Nutrition Affects Flavonoid Biosynthesis in Young Apple (*Malus domestica* Borkh.) Leaves". In: *Plant Biology* 7.6, pp. 677–685. DOI: 10.1055/s-2005-872989.

Surjadinata, B. B. and L. Cisneros-Zevallos (2012). "Biosynthesis of phenolic antioxidants in carrot tissue increases with wounding intensity". In: *Food Chemistry* 134.2, pp. 615–624. DOI: 10.1016/j.foodchem.2012.01.097.

Suzuki, T., Y. Honda, and Y. Mukasa (2005). "Effects of UV-B radiation, cold and desiccation stress on rutin concentration and rutin glucosidase activity in tartary buckwheat (*Fagopyrum tataricum*) leaves". In: *Plant Science* 168.5, pp. 1303–1307. DOI: 10.1016/j.plantsci.2005.01.007.

Syamaladevi, R. M., S. S. Sablani, J. Tang, J. Powers, and B. G. Swanson (2011). "Stability of Anthocyanins in Frozen and Freeze-Dried Raspberries during Long-Term Storage: In Relation to Glass Transition". In: *Journal of Food Science* 76.6, E414–E421. DOI: 10.1111/j.1750-3841.2011.02249.x.

Tattini, M., L. Guidi, L. Morassi-Bonzi, P. Pinelli, D. Remorini, E. Degl'Innocenti, C. Giordano, R. Massai, and G. Agati (2005). "On the role of flavonoids in the integrated mechanisms of response of *Ligustrum vulgare* and *Phillyrea latifolia* to high solar radiation". In: *New Phytologist* 167.2, pp. 457–470. DOI: 10.1111/j.1469-8137.2005.01442.x.

Taulavuori, K., V. Hyöky, J. Oksanen, E. Taulavuori, and R. Julkunen-Tiitto (2016). "Species-specific differences in synthesis

of flavonoids and phenolic acids under increasing periods of enhanced blue light". In: *Environmental and Experimental Botany* 121, pp. 145–150. DOI: 10.1016/j.envexpbot.2015.04.002.

Tegelberg, R. and R. Julkunen-Tiitto (2001). "Quantitative changes in secondary metabolites of dark-leaved willow (*Salix myrsinifolia*) exposed to enhanced ultraviolet-B radiation". In: *Physiologia Plantarum* 113.4, pp. 541–547. DOI: 10.1034/j.1399-3054.2001.1130413.x.

Tsormpatsidis, E., M. Ordidge, R. Henbest, A. Wagstaffe, N. Battey, and P. Hadley (2011). "Harvesting fruit of equivalent chronological age and fruit position shows individual effects of UV radiation on aspects of the strawberry ripening process". In: *Environmental and Experimental Botany* 74, pp. 178–185. DOI: 10.1016/j.envexpbot.2011.05.017.

Uleberg, E., J. Rohloff, L. Jaakola, K. Trôst, O. Junttila, H. Häggman, and I. Martinussen (2012). "Effects of Temperature and Photoperiod on Yield and Chemical Composition of Northern and Southern Clones of Bilberry (*Vaccinium myrtillus* L.)" In: *Journal of Agricultural and Food Chemistry* 60.42, pp. 10406–10414. DOI: 10.1021/jf302924m.

Vogt, T. and P. G. Gul (1994). "Accumulation of flavonoids during leaf development in *Cistus laurifolius*". In: *Phytochemistry* 36.3, pp. 591–597. DOI: 10.1016/s0031-9422(00)89780-0.

Wang, D., J. Lu, A. Miao, Z. Xie, and D. Yang (2008). "HPLC-DAD-ESI-MS/MS analysis of polyphenols and purine alkaloids in leaves of 22 tea cultivars in China". In: *Journal of Food Composition and Analysis* 21.5, pp. 361–369. DOI: 10.1016/j.jfca.2008.01.002.

Wu, X., L. Gu, R. L. Prior, and S. McKay (2004). "Characterization of Anthocyanins and Proanthocyanidins in Some Cultivars of *Ribes*, *Aronia*, *Sambucus* and Their Antioxidant Capacity". In: *Journal of Agricultural and Food Chemistry* 52.26, pp. 7846–7856. DOI: 10.1021/jf0486850.

Yonekura-Sakakibara, K., A. Fukushima, R. Nakabayashi, K. Hanada, F. Matsuda, S. Sugawara, E. Inoue, T. Kuromori, T. Ito, K. Shinozaki, et al. (2011). "Two glycosyltransferases involved in anthocyanin modification delineated by transcriptome independent component analysis in Arabidopsis thaliana". In: *The Plant Journal* 69.1, pp. 154–167. DOI: 10.1111/j.1365-313x.2011.04779.x.

Zandalinas, S. I., C. Sales, J. Beltrán, A. Gómez-Cadenas, and V. Arbona (2017). "Activation of Secondary Metabolism in Citrus Plants Is Associated to Sensitivity to Combined Drought and High Temperatures". In: *Frontiers in Plant Science* 7. DOI: 10.3389/fpls.2016.01954.

Zhang, J., M. B. Satterfield, J. S. Brodbelt, S. J. Britz, B. Clevidence, and J. A. Novotny (2003). "Structural Characterization and Detection of Kale Flavonoids by Electrospray Ionization Mass Spectrometry". In: *Analytical Chemistry* 75.23, pp. 6401–6407. DOI: 10.1021/ac034795e.

Zheng, J., B. Yang, V. Ruusunen, O. Laaksonen, R. Tahvonen, J. Hellsten, and H. Kallio (2012). "Compositional Differences of Phenolic Compounds between Black Currant (Ribes nigrumL.) Cultivars and Their Response to Latitude and Weather Conditions". In: *Journal of Agricultural and Food Chemistry* 60.26, pp. 6581–6593. DOI: 10.1021/jf3012739.

Zietz, M., A. Weckmüller, S. Schmidt, S. Rohn, M. Schreiner, A. Krumbein, and L. W. Kroh (2010). "Genotypic and Climatic Influence on the Antioxidant Activity of Flavonoids in Kale (Brassica oleracea var.sabellica)". In: *Journal of Agricultural and Food Chemistry* 58.4, pp. 2123–2130. DOI: 10.1021/jf9033909.

Peer-reviewed article.
Published on-line on 2017-10-09.
Edited by: Pedro J. Aphalo.