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Julkunen-Tiitto, Riitta

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Assessing the response of plant flavonoids to UV radiation – an overview of appropriate techniques

Riitta Julkunen-Tiitto[1], Nikolaos Nenadis[2], Susanne Neugart[3], T Matthew Robson[4], Giovanni Agati[5], Jouko Vepsäläinen[6], Gaetano Zipoli[7], Line Nybakken[8], Barbro Winkler[9] and Marcel AK Jansen[10]

[1] *University of Eastern Finland, Yliopistokatu 7, 80101 Joensuu, Finland*
[2] *Aristotle University of Thessaloniki, 541 24 Thessaloniki, Greece*
[3] *Leibniz-Institute of Vegetable and Ormamental Crops Grossbeeren, 14979 Grossbeeren, Germany*
[4] *University of Helsinki, 00014 University of Helsinki, Finland*
[5] *CNR-Institute of Applied Physics, 50019 Sesto Forentino, Florence, Italy*
[6] *University of Eastern Finland, Yliopistonranta 1E, 70211 Kuopio, Finland*
[7] *CNR-Institute of Biometeorology, Via Caproni 8, 50144 Florence, Italy*
[8] *Norwegian University of Life Sciences, 1432 Ås, Norway*
[9] *Helmholtz Zentrum München, 85764 Neuherberg, Germany*
[10] *University College Cork, Enterprise Centre Rm 106a, Distillery Field, Cork, Ireland*

Abstract

Flavonoids are a large group of plant secondary metabolites that are present in most plants, and are vital for plant growth, development and protection. Among the many functions of these compounds is their contribution to stress amelioration. The accurate identification and quantification of total or individual flavonoids in plants exposed to stressful conditions (e.g. ultraviolet radiation) is challenging due to their structural diversity. The present review provides the up to date knowledge and highlights trends in plant flavonoid analysis. The review covers all steps from the field to the laboratory, focussing on UV-B effects on flavonoids, and identifying critical issues concerning sample collection, pre-treatment, ~~and~~ extraction techniques and quantitative or qualitative analysis. A well-planned sampling and sample prehandling strategy is vital when capturing organ, tissue and developmental-stage dependent changes in flavonoids, as well as the dynamic changes due to time of UV-exposure and diurnal or seasonal parameters. A range of advanced extraction and purification techniques can facilitate the quantitative recovery ~~transfer~~ of flavonoids ~~to solvents~~. The advantages and disadvantages of analytical methods, including chromogenic assays, liquid and thin-layer chromatography, mass spectrometry, NMR detection, and non-destructive *in situ* fluorescent analysis need to be consciously evaluated in the context of the specific biological question posed. Thus, no one method can be applied to every single study of flavonoid. The message of this review is that researchers will need to carefully consider the biological process that they intend to study, and select an analytical method that optimally matches their specific objectives.

Key Words: Flavonoid extraction, plant flavonoids, qualitative and quantitative analyses, sampling and sample prehandling, ultraviolet radiation

Abbreviations:

APCI	Atmospheric prssire chemical ionization
ASE	Accelerated solvent extraction
BME	Ball-mill extraction
Chl	Chlorophyll
ChlF	Chlorophyll fluorescence
ChlFES	Chlorophyll fluorescence excitation screening
CID	Collision-induced dissociation
CTLS	Constrained total-line-shape
DMACA	Dimethylcinnamylaldehyde
DMSO	Dimethylsulfoxide
2D-LC	Two-dimensional liquid chromatography
ESI	Electrospray ionization
^{1}H-^{1}H COSY	Correlation spectroscopy
^{1}H-^{13}C HMBC	Heteronuclear single multiple bond correlation
^{1}H-^{13}C HSQC	Heteronuclear single quantum coherence
HPTLC	High performance thin-layer chromatography
HSCCC	High-speed counter-current chromatography
HPLC	High-performance liquid chromatography
HPLC-DAD	High-performance liquid chromatography- diode array detection
HPLC-NMR	High-performance liquid chromatography- Nuclear magnetic resonance
HPLC-NMR-MS	High-performance liquid chromatography- Nuclear magnetic resonance-Mass spectrometry
LC	Liquid chromatography
LEDs	Light emitting diodes
MAE	Microwave assisted extraction
MALDI	Matrix-assisted laser desorption ionization
MPa	Mega Pascal
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
ODS	Octadecylsilyl
RP	Reversed phase
RSM	Response surface methodology
SFE	Supercritical fluid extraction
SFE-CO_2	Supercritical fluid extraction assisted by carbon dioxide
SS	Solid state
TLC	Thin-layer chromatography
TOF	Time of flight
TPC	Total phenolic content
TSP	Trimethylsilyl propanoic acid sodium salt
UAE	Ultrasound assisted extraction
UHPLC	Ultra-high pressure liquid chromatography

UMAE	Ultrasound microwave assisted extraction
UV	Ultraviolet
UV-A	Ultraviolet A radiation
UV-A PAM	Ultraviolet A radiation pulse-amplitude modulation
UV-B	Ultraviolet B radiation

Introduction

Flavonoids are a large (over 10.000 identified structures) group of plant secondary metabolites which are present in most plant tissues (e.g. Brunetti et al., 2013, Fig. 1.). Biosynthetically, the flavonoids have a mixed origin being derived from the shikimate and polyketide (acetate) pathways (Harborne, 1989). They are characterized by two aromatic rings that are linked into a three-carbon aliphatic chain (C_6-C_3-C_6), which in turn is condensed to form an oxygen-containing pyran ring. Flavonoids are separated into several subclasses, namely flavones, flavanones, flavonols, flavanonols, flavan-3-ols, chalcones, dihydrochalcones, anthocyanidins, isoflavones, aurones and bioflavonoids, and occur mostly as glycosylated compounds (Crozier et al., 2006). Furthermore, acylation of flavonoid glycosides occurs in most plant species (Calderon-Montano, et al., 2011)

Flavonoids are vital for plant growth, development and protection. These compounds can act as antioxidants, modulators of enzymatic activity, allelopathic agents, UV-absorbing sunscreens, insect attractors or repellents, nectar guides, probing stimulants, viral, fungal, and bacterial protectants, modulators of nodulation in leguminous plants and attractants for pollen germination (e.g. Crozier et al., 2006). The important role of flavonoids in ameliorating various environmental stress conditions has long been recognized (e.g. Roberts & Paul 2006, Pollastri & Tattini, 2011, Brunetti et al., 2013).

As a consequence of stratospheric ozone layer depletion, more solar ultraviolet-B radiation (UV-B, 280-315 nm) reaches the ground, triggering flavonoid accumulation in a variety of plants. Flavonoids can screen harmful UV-B radiation and especially scavenge reactive oxygen species (ROS) formed under stressful conditions, thus preventing oxidation of vital biomolecules (e.g. Agati et al. 2012, Kotilainen et al. 2008, Agati and Tattini, 2010). Over the last three decades, a large number of studies have shown how flavonoid metabolism is affected by UV-B enhancement or exclusion, as well as interactions with other environmental stressors (e.g. Zhang & Björn, 2009, Schreiner et al., 2012). However, the accurate identification and quantification of the concentrations of individual flavonoid compounds under various environmental conditions critically depends on appropriate methodology (analytical and preparative). Appropriate methods are essential to obtain a better understanding of the

role of flavonoids in plant ecophysiology and food chemistry, to facilitate the search for new bioactive compounds but also in quality control and environmental risk studies.

In phytochemical research, validation of results is often challenging due to the chemical and biochemical instability of compounds and their structural diversity. These characteristics affect polarity, solubility and crosslinking, so consequently determine their extractability. Most flavonoids are regarded as moderately stable under various pre-handling and analytical conditions (e.g. Markham 1982, Keinänen & Julkunen-Tiitto 1996, Julkunen-Tiitto & Sorsa 2001). Nevertheless, in order to achieve accurate and reproducible quantification and identification of flavonoids, optimised methodologies for sample pre-handling and extraction, as well as sensitive and selective analytical methods are required. Here, we review current knowledge and recent trends in plant flavonoids analysis, especially focussing on UV-B induced changes in flavonoid profile. The review assesses pre-handling, extraction and analysis methods, and highlights points that are crucial to determining the validity of the results.

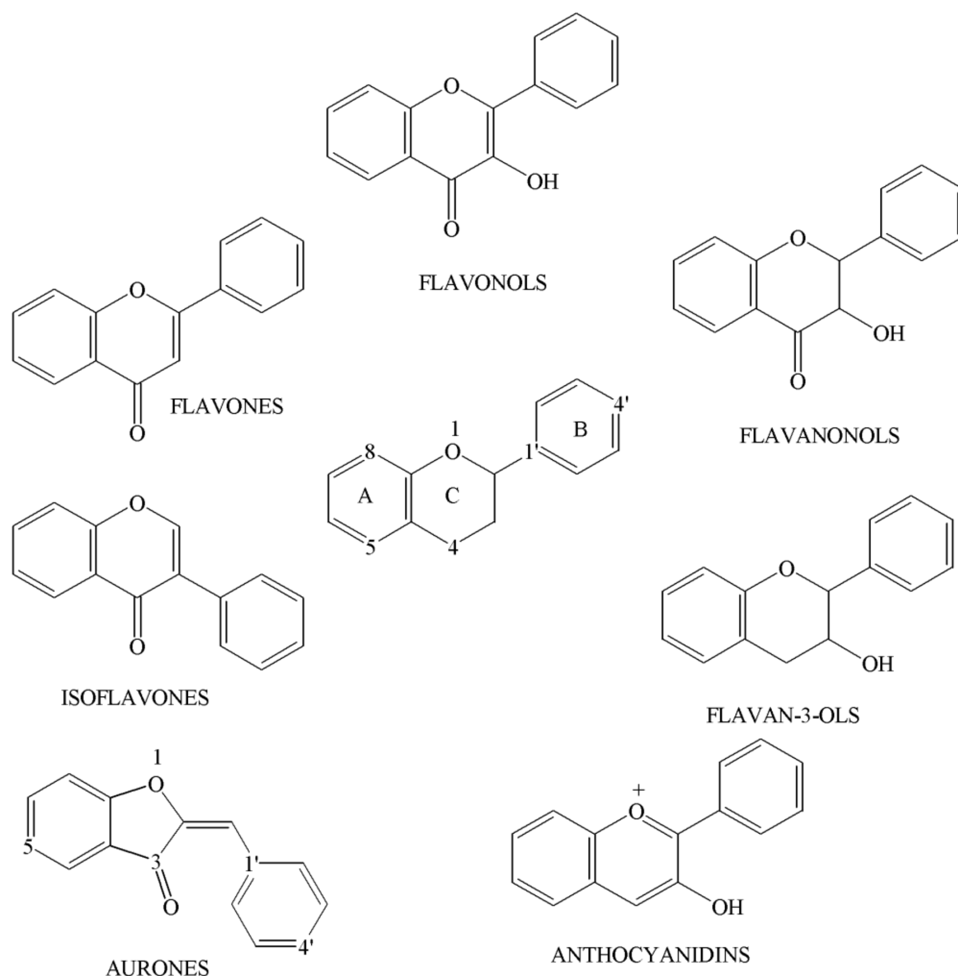

Fig. 1. Categories of natural flavonoids and numbering.

Sampling plant material; choice of plant tissue

Many studies aim to compare flavonoids from plants that were exposed to different environmental conditions, such as UV-B. This requires the use of equivalent, and representative plant tissues. This is not straightforward, as many environmental stressors alter plant development and morphology (Potters et al., 2007). Many ecophysiological studies compare measurements of mature leaves, sampled from two-thirds up from the base of the plant. Thus, the first (i.e. oldest or proximal) and youngest leaves (i.e. terminal or distal) usually are not sampled to avoid any potential effect of development on the flavonoid profile (Laitinen et al. 2002). Besides foliage age, a plant's age is also important when assessing the flavonoid profile of leaves, stems or roots (e.g. de Lucia et al., 1992;

Julkunen-Tiitto et al. 1996, Warren et al., 2002, Laitinen et al. 2002, Keski-Saari and Julkunen-Tiitto, 2003a). Moreover, leaves need to be consistently sampled from the same side of each plant to assure similar exposure to sunlight. Rousseaux et al. (2004) demonstrated that flavonoid profiles in leaves from the north and south sides of *Nothofagus antarctica* (Forster) Oerst were different, and also changed differently upon solar UV manipulation.

Flavonoid content also varies within individual leaves, stems or roots. In grasses, flavonoids accumulate during leaf expansion. However, this is not a homogenous process. Higher levels of flavonoids accumulate at the leaf tip which receives more radiation than the base (Cartelat et al., 2005). Changes in flavonoid profile during leaf expansion are genotype-specific. For instance, in silver birch (*Betula pendula* Roth) seedlings the epidermal UV absorbance attributed to flavonoids (Morales et al., 2011) is higher in expanding than in mature leaves. Similarly, *Zea mays* (L) flavone content is highest in expanding leaves (Casati and Walbot, 2005). In contrast, *Quercus petraea* (Mattuschka) Liebl. flavonoid concentrations increase on a leaf area basis during leaf expansion and remain stable over the growing season, even once senescence has commenced (Louis et al., 2009). Laitinen et al. (2002) reported that concentrations of flavonoid aglycones decreased but flavonoid-glycoside content increased with the phenological advance from mature buds to young leaves in 30-year-old *B. pendula* trees. A study of *B. pubescens* ssp. *Czerepanovii* (Ehrh.) (Keinänen et al. 1998) revealed a large increase in flavonols with development from cotyledons to the first and second true leaves. These complex developmental changes in flavonoid profile are difficult to distinguish from changes driven by seasonal changes in solar UV and/or short-term changes in the weather. This situation is aggravated by the fact that many environmental stressors also impact on plant development (Potters et al., 2007), potentially leading to “indirect” changes in flavonoid profile. Given these complexities, the development of a sampling strategy is a critical component of comparative flavonoid analysis.

Sampling plant material: UV-B exposure and sampling time

Accumulation and loss of flavonoids are time-dependent processes. Yet, the rate of response of leaf flavonoids to changes in solar radiation is still largely unknown. It appears that the bio-accumulation of flavonoids is quite responsive to an increase in UV, unlike the decrease in flavonoids following a decrease in the UV-dose (Sullivan et al., 2007). Accumulation responses also depend on the season; in *B. pendula*, early and late season UV-response trends are distinguishable, and a

significant interaction between UV-treatment and sampling time was discerned (Kotilainen et al., 2010). Another temporal factor is the time of day. Diurnal changes in UV-screening compounds have been reported in *Hordeum vulgare* (L.) and *Glycine max* (L.) Merr. leaves (Sullivan et al., 2007), and these are thought to be related to the need for increased protection around solar noon. UV-A PAM measurements of *Verbascum thapsus* (L.) and *Oenothera stricta* (L.), native Hawaiian plants acclimated to high solar UV and of *Vicia faba* (L.) growing in pots, showed that attenuation of UV transmittance was at its lowest level at predawn and sunset and at its highest near solar noon (Barnes et al., 2008). These diurnal patterns in epidermal absorbance were more pronounced in young compared to old leaves (Flint and Caldwell, 2008). Shade eliminates the diurnal changes in some species but not in others (Veit et al., 1996; Flint and Caldwell, 2008), and in some species diurnal variations in UV-screening are absent altogether (Cartelat et al., 2005). It has not been conclusively shown whether such diurnal changes are regulated by higher photosynthetically active radiation (PAR) (Barnes et al., 2008; Flint and Caldwell, 2008) or UV (Veit et al., 1996) at solar noon or by an internal clock. Chalcone synthase, a key enzyme in the flavonoid biosynthetic pathway, exhibits diurnal changes in activity under artificial light (without UV) conditions in laboratory-grown plants (Peter et al. 1991). However, increases in UV-screening do not necessarily rely on flavonoid biosynthesis. Flint and Caldwell (2008) reported that diurnal changes in UV-screening were not accompanied by changes in total leaf phenolic content, suggesting that improved screening was provided either by the relocation of flavonoids within the leaf, or possibly conversion between phenolic structures. Thus, sampling for flavonoid analysis should always be done at the same time of the day, whilst the time required to harvest all samples should be kept as short as possible.

Sample handling

Appropriate handling is required to assure that the flavonoid concentration and profile are preserved in the harvested plant material. When plant material is collected, it is imperative that measures are taken to counter flavonoid degradation. If immediate extraction of the intact (fresh) samples is not possible, samples need to be quickly transferred to the laboratory for drying and storage. Drying and storage of dried plant material is commonly used where no high though-put extraction method is available.

Plant material can be dried by either thermal (oven or microwave drying) or non-thermal (air, dry air, vacuum or freeze-drying) techniques. Flavonoid compounds differ in their thermostability. While isoflavonoid glycosides of soy germ flours are stable up to 130 °C (Tipkanon et al. 2011), the anthocyanins of apple pomace are completely degraded by drying temperatures of 50 °C or higher (Heras-Ramírez et al. 2012). Furthermore, the impact of drying on the concentrations of flavonoids or phenolics differs strongly between species. In *Alpinia zerumbet* (L.) the use of thermal drying resulted in the loss of approximately 50% of the total phenolic content, whereas sun- or oven drying of *Etlingeria elatior* (Jack) R.M. Sm. reduced the total phenolic content by up to 91% compared to fresh leaves (Chan et al. 2009). In *Solanum lycopersicum* (L.), *Beta* spp., *Solanum melongena* (L.) and *Colocasia esculenta* (L.) Schott the total phenolic content (TPC) was greatly decreased by oven-drying at 55 ºC, while in carrots subjected to non-thermal freeze-drying no change in TPC was apparent (Hung and Duy 2012). Similarly, heat drying (at 60 and 90 ºC) was deleterious to *Salix purpurea* (L.) leaf flavonoids including naringenin-glycosides, eriodictyol-glycoside, apigenin-glycosides, catechins and condensed tannins (Julkunen-Tiitto and Sorsa 2001). However, in contrast to these findings, drying of *B. pendula* leaves at 80 ºC yielded significantly higher concentrations of quercetin and myricetin glycosides compared with drying at 40 ºC (Keinänen and Julkunen-Tiitto 1996). The same study also showed that freeze-drying at -30 ºC is a better method for preservation of flavonoids than conventional freeze-drying where plant material is pre-frozen and freeze-dried in a drying chamber at +18 ºC. Good preservation of flavonoids in freeze-dried samples was also achieved for the individual flavonoids of *Vitis* spp. skin (de Torres et al. 2010) or *Tuberaria lignosa* (Sweet) Samp. (Pinela et al. 2012). Summarizing the recent literature, thermal drying techniques, including oven drying and sun drying, often decrease both the TPC (Chong and Lim 2012, Zhou et al. 2011) and the contents of individual flavonoids (de Torres et al. 2010). The non-thermal process of freeze-drying appears to be the most suitable drying method for samples for flavonoid analysis, having the least effect on TPC (Chong and Lim 2012). Nevertheless, even freeze-drying has been reported to cause species-specific increases or decreases in TPC (Chan et al. 2009).

Storage conditions of dried plant material are also important for flavonoid preservation. Neither 15 nor 30 days of storage at room temperature changed the TPC of freeze-dried leaves of *Vitex* spp. (Chong & Lim 2012). Likewise, storage of freeze-dried leaves for seven days at room temperature did not affect the TPC for different ginger species. In contrast, the TPC of equivalent air-dried leaves decreased over the same time period (Chan et al 2009). In a systematic study on the importance of

storage conditions, Syamaladevi et al. (2011) measured total anthocyanin content in freeze-dried *Rubus* spp. powder stored at a range of storage temperatures, and for periods up to 378 days. Storage temperatures ranging from -20 down to -80 °C had no influence on the degradation of anthocyanins. In contrast, if raspberry powder was stored at 23°C, all anthocyanins had degraded after 233 days. Unlike the temperature, the moisture content of the powder correlated negatively with the total anthocyanin concentration after storage (Syamaladevi et al. 2011). Freeze-dried powders of *Allium cepa* (L.) containing just 3% moisture could be stored for up to six months without an effect on the concentrations of flavonols and anthocyanins (Pérez-Gregorio et al. 2011). Moraga et al. (2012) reported that storage of freeze-dried *Citrus* x *paradisi* powder at 20°C for three months had no effect on flavanone, flavanone glycosides and quercetin concentrations, irrespective of the relative humidity. However, after six months storage of the freeze-dried powder stored at a relative humidity of 40% or more, the flavonone glycoside concentration decreased sharply, except for the flavonoid didymin whose concentration inceased at a relative humidity of 30% (Moraga et al. 2012). These data show that non-optimal storage can affect both flavonoid concentrations as well as the flavonoid profile. To summarize, in order to preserve flavonoids in biological samples, the water content of freeze-dried powdered samples should be as low as possible. It appears, if samples are well dried, the storage temperature is less important for preservation of flavonoids. Nevertheless, it is recommended to keep the storage time of samples as short as possible, especially for samples that are analysed for their anthocyanin content.

Extracting flavonoids from plant samples

Solvent extraction is a critical process that generates a crude extract enriched in the compounds of interest. However, extracts will also contain interfering substances, and therefore will need to be subjected to further purification in order to obtain accurate qualitative and/or quantitative information about individual metabolites (e.g. Stalikas 2007, Naczk and Shahidi 2004). Flavonoids, particularly when glycosylated, are prone to chemical and biochemical decomposition during extraction, especially if samples are fresh or not properly dried. Therefore, rapid extraction procedures are required to accurately determine the flavonoid profile in living plant tissues. Polar solvents such as water, acetonitrile, ethyl acetate, ethanol or preferably methanol (pure, mixed with water or acidified) are used for solid to liquid extraction of flavonoids (Waksmundzka-Hajnos et al. 2011, Bacci et al. 1999,

Julkunen-Tiitto & Sorsa, 2001, Ming et al. 2012, Liimatainen et al. 2012, Lizana et al. 2009). To facilitate quantitative transfer of flavonoids to the solvents, stirring and/or conventional heating have been employed during maceration. Nowadays, Soxhlet and reflux extractions tend to be replaced by modern fast techniques that exploit other types of energy or even the properties of the solvents under pressure or at their supercritical state (Hartonen et al. 2007, Velicovic et al. 2007, Wang et al. 2008, Xiao et al. 2008). These advanced extraction techniques have several advantages, including that (1) very small samples can be processed, (2) thermo- labile compounds can be recovered, (3) small volumes of solvent suffice, and (4) faster processing has reduced the cost of analysis (Ajila et al., 2011).

Ultrasound assisted extraction (UAE) is a modern technique that exploits the so-called ultrasound induced "cavitation phenomenon" in the sample matrix (Seidi and Yamini 2012). UAE is a relatively fast and low cost technique for the recovery of flavonoids from the different parts of plants, and it can even be coupled to chromatographic techniques in order to speed-up analysis (Velicovic et al. 2007, You et al. 2010, Naşcu-Briciu et al. 2011, Annegowda et al. 2012, Pan et al. 2012). For example, using UAE at room temperature, Martino et al. (2008) were able to recover a series of flavonoid glycosides from *Crataegus monogyna* (Jacq.) dried leaves and flowers at levels comparable, or in certain cases almost 2-fold higher, to those extracted with the aid of Soxhlet or maceration (60 ^{o}C). Moreover, UAE took 5-6-fold less time than traditional techniques. Extraction without heating makes UAE suitable for the recovery of thermolabile flavonoids. Nevertheless, as ultrasound produces heat when used for a sustained period, temperature control is a prerequisite to maintain the integrity of sensitive compounds (Wang and Weller 2006). Furthermore, caution should be taken when selecting UAE conditions, as prolonged applications using highly aqueous solvents (more than 50% content of water) may cause degradation of compounds possibly due to solvent dissociation giving rise to oxidizing free radicals (Shirsath et al. 2012).

Microwave assisted extraction (MAE) is another modern extraction technique. It exploits the penetrating and heating efficiency of microwave irradiation (Wang and Weller 2006). MAE has been successfully used for flavonoid recovery, including the extraction of rutin and isoquercitrin from *Sambucus nigra* (L.) flowers (Waksmundzka-Hajnos et al. 2008a), and hyperin, vitexin and vitexin-2″-O-rhamnoside from *C. monogyna* dried leaves and flowers (Martino et al. 2008). In the latter case, MAE achieved adequate flavonoid recovery with less than 3 minutes of extraction at 60 ^{o}C, which is 20 times faster than UAE. Utilization of a closed MAE vessel apparatus instead of an apparatus working at

atmospheric pressure increases the extraction efficiency by combining heat with pressure, as temperatures beyond the boiling point of the solvents can be achieved. Consequently, the duration of the extraction step can be even shorter, while recovery remains high even for thermolabile compounds (Camel 2001, Biesaga 2011, Biesaga and Pyrzynska 2013). Further improvements in MAE can be achieved by using ionic liquids as solvents, due to their low vapour pressure and their excellent microwave-absorbing ability (Du et al., 2009), or by combining MAE with other extraction techniques. An example of this is ultrasound microwave assisted extraction (UMAE) used by Cheng et al. (2011) for the recovery (87 to 101%) of six *Spatholobus suberectus* (Dunn) flavonoids from dried plants stems. The total flavonoid yield recovered using UMAE was 1.4 and 1.9 times greater than with MAE or UAE, respectively. Furthermore, the amount of solvent used was 2-6 times less and the duration of extraction was only 7.5 minutes instead of 30 minutes with MAE or 1 hour with UAE.

Accelerated solvent extraction (ASE) employs solvents at high temperature (50-200 ^{o}C) and pressure (up to 10-14 MPa) to accelerate mass transfer. ASE can be successfully used in the extraction of different flavonoids (e.g. Richter et al. 1996). Bergeron et al. (2005) compared the effectiveness of ASE with different extraction methods (e.g. conventional hot water and 70% ethanol). ASE at 85 ^{o}C, with water as a solvent, gave the best recovery of flavonoid glycosides from dried *Scutellaria lateriflora* (L.) material. Similarly, ASE at 100 ^{o}C and about 10 MPa, with methanol as solvent, gave a significantly higher yield of catechin and epicatechin from *Vitis* spp. seeds compared with UAE. The extraction time was also shorter (Pineiro et al 2004). The efficiency of ASE, using methanol as a solvent (100 ^{o}C, 6 MPa), is superior to that of Soxhlet extraction, UAE and MAE, for the recovery of flavonoids of *Polygonum aviculare* (L.) foliage (Waksmundzka-Hajnos et al. 2008a). When water is used as a solvent, the addition of a surfactant (e.g. sodium dodecyl sulphate) at a concentration of 0.1-0.2% w/w can further improve recovery of flavonoid glycosides by 2-5 fold, as shown for rutin and quercitrin from the flowers of *Coctus speciosus* (Chang et al., 2011). In this way, a 30 minute recovery protocol with ASE gives a comparable yield to a 6 hour Soxhlet extraction using 80% methanol. Nevertheless, the high temperatures in ASE should be considered when thermolabile compounds are extracted.

Supercritical fluid extraction (SFE) is often used for lipids, essential oils and volatile compounds. However, this technique can also be useful for the isolation of flavonoids yielding extracts of high purity (e.g. Waksmundzka-Hajnos et al. 2011). A modifier (co-solvent) to increase the polarity of flavonoids has to be added to CO_2 in order to facilitate flavonoid extraction (e.g. Lin et al., 1999).

SFE has been found to be a good method to extract naringin from *Citrus* x *paradisi* (Macfad) (Giannuzzo et al. 2003), epicatechin from sweet *Tamarindus indica* (L.) seed coats (Luengthanapol et al. 2004) and baicalin, baicalein and wogonin from *Scutellaria baicalensis* (Georgi.) radix (Lin et al. 1999). In the latter case, utilization of methanol:water (70:30, v/v) instead of methanol as a co-solvent (in a ratio of 3:20 with CO_2) resulted in 2 - 5 times higher recovery of baicalin, baicalein and wogonin (Lin et al., 1999). SFE-CO_2 with 20 % methanol as co-solvent can also give a superior yield and recovery of orotin, orotin-5-methyl ether and licoagrochalcone B from *Patrinia villosa* (Thunb.) Juss. (Peng et al. 2006). Whereas, SFE-CO_2 extraction using ethanol as a co-solvent is considered the best method for the extraction of flavonoids from *Ginkgo biloba* (L.) leaves (Chiu et al. 2002) and *Pueraria lobata* (Willd.) Ohwi roots (Wang et al. 2008).

In addition to the aforementioned techniques, cutting homogenizers (e.g. Laitinen et al. 2002, Naczk and Shahidi 2004, Keski-Saari et al 2005) and more recently micro- or semi-micro scale extraction methods have been developed. These include for example ball-mill extraction (BME), whereby a sample is homogenized while flavonoids are simultaneously dissolved into a solvent. BME can be performed at room, or lower (liquid nitrogen cooling), temperatures. The amount of plant material required is small, 2 to 8 mg depending on flavonoid content. BME has reduced the extraction time to 15 minutes or les, can recover more than 98% of flavonoids and is suitable for fresh and dried leaf, stem and root samples (Kosonen et al. 2012, Nybakken et al. 2011). A variation is mechanochemical-assisted extraction, whereby solid reagents (e.g. Na_2CO_3, $NaHCO_3$) are mixed with the plant material in a ball mill. Under these conditions flavonoids are converted into their polar salts with concomitant homogenization of the plant material. Flavonoid salts can be easily extracted with water and recovered afterwards via acidification of the solution. This technique has been applied in the extraction of homoorientin, vitexin and isovitexin from *Phyllostachys edulis* (Carriere) J. Houz leaves (Xie et al., 2013) and rutin from *Hibiscus mutabilis* (L.) leaves (Xie et al. 2011).

A particular problem that can be encountered when extracting the flavonoids is the presence of covalently bound compounds. Cell-wall bound flavonoids can be recovered using enzymes such as cellulases, pectinases and xylanases or mixtures of these (Puri et al. 2012). Cell-wall degrading enzymes also have high transglycosylation capacity and can, in the presence of glucose, produce a significantly increased flavonoid yield since the less polar aglycones can be extracted after conversion into glycosides (Chen et al. 2011). However, this does distort the specific flavonoid-glycoside profile of the tissue that is being studied.

Regardless of the extraction method selected, parameters such as the extraction time, temperature, pH, solvent composition and the solute to solvent ratio need to be optimised in order to maximize the recovery of flavonoids. Since this is often a cumbersome procedure, mathematical approaches can be used in combination with response surface methodology (RSM) which assists in exploring the contribution of each variable to the extraction efficiency (e.g. Pan et al. 2012).

Purification of flavonoids

Purification of compounds that are normally present in complex plant matrices enables structural studies and identification of a target compound. Several techniques are available to purify compounds, including fast liquid-liquid chromatography known as high-speed counter-current chromatography (HSCCC), solid phase extraction (SPE), and preparative HPLC, and these can also be combined to enhance their overall efficiency (Li et al. 2011, Qu et al. 2012).

HSCCC is used to isolate small numbers of compounds from plant matrices. However, the amount of ground plant powder needed is high, and can vary from 5 g (Liang et al 2011) up to 2 kg (Zhang et al. 2011). The resulting eluents (large volumes of 90-95% ethanol or methanol) are evaporated and extracts are diluted in water. Afterwards, the extracts are further extracted with ethyl acetate (Li et al. 2012, Tang et al. 2011). The solvent system to be used in HSCCC can be determined by calculating the partition coefficient for each target compound. Xie et al. (2011) reported that a solvent system comprising *n*-hexane:ethyl acetate:methanol:water (5:7:5:5, v/v) was most efficient for the purification of flavonoid aglycones such as luteolin and apigenin. In contrast, isolation of kaempferol dirhamnoside required a less organic solvent system made up of ethyl acetate:*n*-butanol:water (4:1:5, v/v). Using HSCCC, it is common to obtain purities greater than 90%. As a consequence, the total mass of compounds isolated varies from less than 40 mg up to 500 mg (Tang et al. 2011) after 8 hours (Wei et al. 2011), depending on the amount of crude extract injected. However, the duration of the isolation can be manipulated by changing the mobile phase after a set point (Wei et al. 2011) or by increasing the flow of the mobile phase (Xie et al. 2011). In conclusion, HSCCC is a suitable method to isolate large amounts of a specific target compounds from plant material.

Another common purification technique is preparative high-performance liquid chromatography (HPLC). Frequently, a gradient of acidified water and methanol or acetonitrile is passed through a reverse phase C_{18} column at a flow rate of up to 5 ml min^{-1} (Li et al. 2011, Abdulmanea et al. 2012,

Fiol et al. 2012). Fractions are collected dependent on the absorption at λ_{max} of the target compounds, at for example 276 nm (Li et al. 2011) or 280 nm (Fiol et al. 2012). A metabolite purity of ~90% can be reached with this method, but several runs are needed to obtain adequate amounts of metabolites for further identification (Fiol et al. 2012).

Solid Phase Extraction (SPE) is a technique that is mainly used for separating compound groups from each other and/or to exclude interfering compounds such as sugars from a sample (Zhang et al. 2011). SPE cartridges differ in size, ranging from just 50 mg of absorbing material to self-packed big cartridges up to 40 cm in length (Jwanny et al. 2012). The absorbing materials may be Sephadex LH-20 (Jwanny et al., 2012), polyamide resin (Wang and Zhang, 2012) or C_{18} silica (Zhang et al. 2011), depending on both the polarity of the targeted phenolics and the cost. The use of cartriges loaded with so called "molecular-imprinted polymers" is a promising development that appears to achieve increased selectivity. These cartridges have been designed using the target metabolite as a template molecule to construct cavities that have a high affinity for the compounds of interest (Pardo et al., 2012). Target phenolic compounds can be eluted from cavities using methanol (Zhang et al. 2011) or ethanol (Wang and Zhang 2012). Eluted fractions often include more than one compound and the elution of different flavonoid classes can vary, which is a disadvantage of this method (Wang and Zhang 2012). However, it is a fast and cheap method to clean or fractionate polyphenolics from plants samples. Moreover, SPE can be coupled with MS measurements or NMR measurements for further structural information (e.g. van der Hooft et al. 2013).

The choice of purification technique will depend on the facilities and expertise available in a particular lab. HSCCC, preparative HPLC and SPE are all suitable for flavonoids but the fractions obtained require further identification using HPLC-MS and/or NMR. The high purity of compounds obtained by HSCCC and preparative HPLC make these techniques preferable for studies of the physic-chemical characteristics of a target compound.

Qualitative and quantitative analyses of flavonoids

Quantitative chromogenic assays

Advantages of "chromogenic assays" for flavonoid analysis include low cost, high speed in comparison to chromatographic techniques, as well as the claim that these assays provide information

on the total flavonoid content. The total flavonoid content cannot easily be acquired using sophisticated techniques (HPLC, GC) due to variable system efficiency and the lack of available standards (He et al. 2008). According to the literature, there is no accepted and universal method for the measurement of total flavonoid content (Denni and Mammen, 2012, Ho et al., 2012), even despite the improved but cumbersome protocol proposed by He et al. (2008). This problem is probably due to the great number of natural flavonoids and the diversity of their structures (e.g. Price et al., 1978, Zhuang et al., 1992, Woisky and Salatino, 1998, Chang et al., 2002).

The protocols developed for flavonoid quantification exploit the use of certain reagents applied to visualise flavonoid classes after thin layer chromatography (TLC) or to cause a shift in the ultraviolet/visible (UV-Vis) spectra of compounds (e.g. Markham, 1982, Viswanathan et al., 2000, Ho et al., 2012). Colour development is due to the formation of a complex between flavonoids and certain metal ions (Woisky and Salatino, 1998, Viswanathan et al., 2000) or adducts (Nagy and Grancai, 1996, Sun et al., 1998). Chromogenic assays have been widely used in the past, but recent re-evaluations of some of the protocols have raised concerns about what is really measured (Papoti et al., 2011, Denni and Mammen, 2012, Ho et al., 2012). A frequently employed method is based on the complexation of flavonoids with Al(III) ions, alone or in the presence of sodium acetate, an acid or sodium nitrite in alkaline environment (Woisky and Salatino, 1998, Chang et al., 2002, Cvek et al., 2007). When plant extracts are mixed with just Al(III) ions, usually in alcoholic media, then complex are formed with flavonols and flavones, resulting in the formation of stable colored (yellow) products (Ho et al., 2012). However, the method can cause an overestimation of flavonoid content by including non-flavonoid compounds bearing a catechol moiety such as caffeic acid and oleuropein (Papoti et al., 2011). Moreover, colour reactions also depend on the flavonoid structure, with 3-hydroxy-4-keto> 5-hydroxy-4-keto > ortho-dihydroxy group (Ho et al., 2012). This sort of interference is quite small in the presence of sodium acetate (Ho et al., 2012) and is absent in an acidic environment (Papoti et al., 2011), however, the complexes of Al(III) with ortho-dihydroxy phenolic compounds are labile in acid (Markham, 1982). Using a series of commercially-available flavonoid aglycones, Denni and Mammen (2012), showed that complexes of flavonoid aglycones (except myricetin, quercetin and kaempferol, λ_{max} = 423-426 nm) with Al(III) present a λ_{max} significantly different (330-411 nm) from that used for quantification purposes (415 nm). Moreover, the presence of water-organic solvent mixtures, typically used for extraction of flavonoids, has been reported to affect the colour shift upon complexation with Al(III) ions (Markham, 1982) or even to decrease colour formation as in the case of the vanillin assay

(Hagerman and Butler 1994). Consequently, the significance of the results obtained using Al(III) is questionable, especially for samples with an unknown flavonoid composition. In addition to Al(III), other metal ions (molybdenum, antimony and bismuth salts) have also been used for flavonoid detection (e.g. Viswanathan et al. 2000).

Another commonly used chromogenic assay determines flavanones that are detected through their reaction with 2,4-dinitrophenylhydrazine in the presence of sulphuric acid to form phenylhydrazones that absorb at 486 nm (Nagy and Grancai, 1996). Quantification of flavan-3-ols, such as catechins and related compounds, is based on their reaction with vanillin or even dimethylcinnamyldehyde (DMACA) leading to coloured products that absorb at 500 and 632 nm, respectively (e.g. De Pascual-Teresa et al., 1998, Sun et al., 1998, Correia et al. 2006, Glavnik et al. 2009). These products are formed by condensation reactions at the C-6 and/or C-8 carbon atoms on the A-ring, which may occur more easily in the case of flavan-3-ols due to the absence of the electron withdrawing 4-keto group (Desphande et al., 1986). Phenolics that bear ortho-substitution do not react in this way (Desphande et al., 1986), thus, interference caused by the concomitant presence of common phenolic acids is avoided. Of these two assays, the one utilizing DMACA is proposed to be more sensitive than the vanillin assay, which is negatively affected by the type and concentration of the acid used, temperature , light, water content, and interfering substances such as ascorbic acid and chlorophyll (Broadhurst and Jones, 1978, Sun et al., 1998).

To resolve shortcomings in the aforementioned protocols, He et al. (2008) developed a sodium borohydride/chloranil-based assay for the quantification of total flavonoid content. The chemistry of the proposed protocol involves reduction of flavonoids bearing a 4-carbonyl group and oxidation of the C-ring of the reduction products into anthocyanins which are eventually quantified through an adduct formation with vanillin at 490 nm (Fig. 2). The resulting assay aims to detect all types of flavonoids, with a detection limit of approximately 0.1 mM and, apparently, without interference from phenolic acids. However, the measurement takes long time (~ 2.5 h), the procedure is cumbersome and uses toxic chloranil. Furthermore, such a “total flavonoid” assay may not be that valuable given that only some, not all, flavonoid-glycosides are affected by UV-B radiation. Consequently, chromogenic assays with selectivity for individual categories of flavonoids may be more appropriate for UV-acclimation studies.

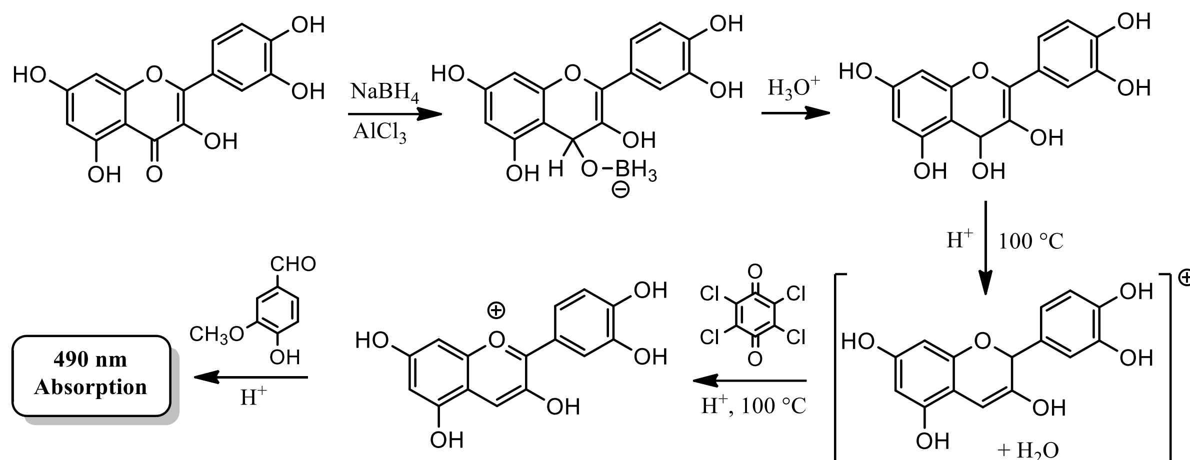

Fig. 2. The chemistry involved in total flavonoid analysis according to He et al. (2008): (1) Flavonoids with a 4-carbonyl group were reduced to flavan-4-ols using sodium borohydride catalyzed with aluminum chloride. (2) Flavan-4-ols were oxidized to anthocyanins by chloranil in acetic acid solution. (3) Anthocyanins were reacted with vanillin in concentrated hydrochloric acid to yield a characteristic absorbance at 490 nm.

Thin-layer chromatography

Chromatographic techniques can generate qualitative and quantitative information on individual flavonoid glycosides. TLC is still commonly applied in flavonoid analysis due to its simplicity and speed, allowing a large number of samples to be analyzed within a short time. TLC analysis of flavonoids is mostly carried out on plates with silica as the stationary phase, though applications with plates covered with cellulose, polyamide or silica bonded with e.g. cyanopropyl or octadecyl groups have also been used (Conde et al., 1992, Karuza et al., 1996, Hawryl et al., 2002, Kagawa et al., 2004). In this way, it is possible to carry out separations based on the adsorption of the target compounds to the stationary phase or their differential partition between the stationary and the mobile phase, affecting noticeably, separation selectivity. Numerous solvent systems have been utilized for the separation of flavonoids using TLC. Chloroform-methanol mixtures (e.g. 96:4, v/v) are used for the separation of flavonoid-aglycones, such as apigenin, luteolin, and quercetin. Highly methylated or acetylated flavones and flavonols can also be separated using chloroform-methanol (e.g. 15:1, v/v) chloroform-methanol) on silica gel precoated plates. Whereas, flavonoid glycosides can be separated using a mixture ethyl acetate-formic acid-glacial acetic acid-water (100:11:11:26, v/v/v/v) (Marston and Hostettmann, 2006).

Separated flavonoids are usually detected on the dried TLC plate using 366-nm excitation; depending on the structure, dark yellow, green, or blue fluorescent spots are visible. In order to improve detection, fluorescence can be enhanced by spraying various reagents onto the plate. Among the most popular of these are natural product-polyethylene glycol, 1% ethanol or methanol aluminum-chloride ($AlCl_3$) solution (e.g. Medic-Šaric et al., 2008). Despite the fact that TLC analysis should provide qualitative information, quantitative data can also be obtained with the aid of a densitometer. Especially after spraying with visualization reagents, flavonoids can be detected down to 20 ng (e.g. Hiermann and Bucar, 1994). Additional information on the identity of compounds can be acquired by equipped the densitometer with diode array detector, in combination with reference compounds or through on-line coupling of TLC with e.g. Fourier transform infrared spectrometry or mass spectrometry (Waksmundzka-Hajnos et al., 2008b).

To achieve higher separation efficiency, high-performance TLC (HPTLC) plates can be used in combination with automatic sample application (e.g. CAMAG TLC sampler) that accurately deposits reproducible volumes of extracts and standards. HPTLC uses very thin layers of sorbent with small and uniform particle size. The developing front is also shorter compared with conventional TLC. These factors result in faster separation, reduced zone diffusion, better separation efficiency, lower detection limits, and the ability to apply more samples per plate (Sherma and Fried, 2003). Nevertheless, as plant extracts are complex mixtures of metabolites, effective separation of all compounds cannot be expected. To address this, modes such as unidimensional multiple development and two-dimensional development have been introduced and applied to flavonoid analysis. In the first scenario, progressive developments of a chromatographic plate are carried out using different elution systems, with a drying step in between each development (e.g. Gocan et al., 1996). In the second scenario a single sample is applied in the corner of a plate, and the layer is developed first in one direction, and then with a different eluent in the other direction. This way, very complex mixtures can be separated successfully (e.g. components of a plant extract) over the entire surface layer (Hawryl and Soczewinski, 2001).

Flavonoid analysis using high performance chromatography coupled to diode array detection

High performance chromatography (HPLC) coupled with photodiode array detection (PDA) is the method of choice in both qualitative and quantitative analyses, as well for preparative separation of all classes of flavonoids, and it is applied very broadly. Successful HPLC analysis depends on the

separation column stationary phase, optimized eluent composition, and the eluent gradient. The most frequently employed columns are based on octadecylsilyl- bonded ODS (RP-18, or C_{18}) phases (e.g. Marston and Hostettmann 2006) and are well end-capped (residual silanol groups decrease separation selectivity) (Cuyckens and Claeys 2004). Crozier et al. (1997) tested different stationary phases and showed that ODS-Hypersil and LiChrospher RP-18 are superior to Nova-Pak, Symmetry and Genesis columns especially for flavonoid glycosides, while other studies indicate ODS-Hypersil to be equally good for aglycones (e.g. Peltonen et al. 2006, Räisänen 2008). Flavonoid aglycones are eluted later than flavonoid glycosides and replacement of the hydroxyl-substituent with a methyl- or acetyl-substituent markedly increases the elution time (e.g. Crozier et al. 1997, Julkunen-Tiitto and Sorsa 2001, Keski-Saari et al. 2003b, Keski-Saari et al. 2005, Graglia et al. 2001, Nyman and Julkunen-Tiitto 2000). The most common elution order for flavonol *O*-glycosides is galactosides < glycosides/glycuronides < arabinosides < rhamnosides (e.g. Keinänen and Julkunen-Tiitto 1998, Marz et al. 2009, Fares et al. 2010, Persson et al. 2012, Lavola et al. 2013). The position of glycosylation will affect the retention time. Flavone *C*-glycosides (e.g. apigenin 8-*C*-glycoside) elute faster than flavone *O*-glycosides (apigenin 7-*O*-glycoside), and likewise flavone 8-*C*-glycosides elute before flavone 6-*C*-glycosides (Marston and Hostettmann 2006). Moreover, flavanones have a shorter retention time compared with their corresponding flavones (Marston and Hostettmann 2006). Generally, temperatures from 25 to 30°C are used for the analyses of flavonol aglycones and flavonol glycosides, and a constant column temperature enhances reproducibility (e.g. Zhang et al. 2010, Neugart et al 2012).

The most common solvents used to separate flavonoids using HPLC are acetonitrile-water or methanol-water mixtures modified with *o*-phosphoric, acetic acid, citric acid, trifluoroacetic acid or formic acid (e.g. Nyman and Julkunen-Tiitto 2000, Marston and Hostettmann 2006, Waksmundzka-Hanjos et al. 2011). In addition, different buffer-eluents, such as acetate and formate are used at low pH (Waksmundzka-Hanjos et al. 2011). Especially, anthocyanins are stable at low pH (1-3) (Carbita et al. 2000). A small amount (1.5 to 1.8 %) of tetrahydrofuran in the mobile phase has proven to be a powerfull modifier of most flavonoids, increasing peak resolution and shortening retention times (e.g. Meier et al. 1988, Crozier et al. 1997, Julkunen-Tiitto and Sorsa 2001).

Most flavonoid work using HPLC has been done at a maximum pressure of 40 MPa, often with 6 to 20 cm columns with an inner diameter greater than 3 mm and particle size of 3 to 5 µm. Recently-developed ultra high pressure LC (UHPLC) can operate at up to 120 MPa with a column diameter of 2.1 mm and a particle size of just 1.7 µm. This markedly enhances sensitivity, improves resolution,

increases peak efficiency, reduces solvent requirements and speeds up analyses of the flavonoids compared with conventional LC (e.g. Baranowska & Magiera 2011), and can be coupled to mass spectrometry (MS) (e.g. Motilva et al. 2013). Klejdus et al. (2008) have developed UHPLC methods for several plant species including *Trifolium pratense* (L.), *Glycine max* (L.) Merr., *Pisum sativum* (L.), and *Ononis spinosa* (L.), and were able to separate 10 isoflavonoids within 1.5 minutes using a linear elution gradient of aqueous acetic acid with pure methanol and a chemically-bonded stationary C_{18} phase. Similarly, seven different flavanols from *Camellia sinenesis* (L.) Kuntze were successfully separated within less than 2 minutes with an endcapped, monomeric C_{18} column (Guillarme et al. 2010). Nevertheless, challenging may be in the future the efficiency of newly developed fused-core particle columns with a particle size of 2.6 µm claimed to improve separation, shortening the time of analysis and "bridging" partially the gap between UHPLC and typical HPLC instruments (e.g. http://www.hplc.eu/halo.htm). For very complex plant samples two-dimensional LC (2D-LC) has been introduced (Motilva et al. 2013, Kalili et al. 2009). 2D-LC has been used to separate of *C. sinensis* leaf phenolics (Scoparo et al. 2012) and *Malus* spp. (Red Starking variety) phenolics (Kalili et al. 2009). The optimized 2D-LC technique employs size exclusion and reverse phase BEH-C_{18} chromatography, and can analyze more than 80 closely-related compounds including several flavonols, their glycosides and flavan-3-ols (Scoparo et al. 2012). Off-line 2D-LC (HILIC and RP-LC) has proven to be suitable for the analyses of a complex mixture of apple proantocyanidins (monomers to higher oligomers) (Kalili et al. 2009).

Flavonoids in general can be detected at a wavelength of 280 nm, even though those compounds absorbing at this wavelength are not exclusively for flavonoids(Fiol et al. 2012, Mascherpa et al. 2012). Additionally, various flavonoid classes have different absorption maxima that can be used for identification. These arise from characteristic absorption bands, Band I (B-Ring) with maxima between 300-550 nm and Band II (A-Ring) with maxima between 240-285 nm (Guiyev et al. 2004). Absorption at 340 nm has been used for quantifying flavones (Justesen et al. 1998), at 290 nm for flavanones, at 280 nm for flavan-3-ols (proanthocyanidins) (Mullen et al. 2007), at 365 nm for flavonols (Mullen et al. 2007) but also 370 nm (Schmidt et al. 2010), at 260 nm for isoflavonoids (Tipkanaon et al. 2011) and at 520 nm for anthocyanins (Mullen et al. 2007, Huyskens-Keil et al. 2007, Da Sila et al. 2012). The glycosylation of flavonoids can affect their absorption wavelength, as shown in *Allium* pp. where flavonol glycosides were quantified at 350 nm (Bonaccorsi et al. 2005). Acylation of flavonoid glycosides leads to further changes in the absorption maximum (Olsen et al. 2009,

Schmidt et al. 2010). However, spectra (190-600 nm) recorded using diode arrays detectors are often used in flavonoid analyses allowing multiple absorption maxima to be identified (e.g. Mascherpa et al. 2012).

Identification of flavonoids using mass spectrometry

In addition to HPLC-DAD analyses, mass spectrometry is a modern tool for the analyses of flavonoids (Fig.3.). Many glycosides from specific plant groups have been identified by mass spectrometry in particular species, e.g. *Brassica* (Schmidt et al. 2010, Cartea et al 2011), herbs (Justesen 2000, Boros et al. 2010), Chinese medicinal plants (Lai et al. 2007, Cheng et al. 2011) and trees or perennial plants (e.g. Lorenz et al. 2011, Rummukainen et al. 2007, Keski-Saari et al. 2003b, Tegelberg et al. 2001). The identification of flavonoids can give further information on the effect of UV on different structures (Neugart et al. 2012). In addition to the often-used soft ionization technique ESI (Harnly et al. 2007, Gouveia et al. 2013), APCI and MALDI are suitable ionization techniques for phenolics (Harnly et al. 2007, Ieri et al. 2012). Flavonoids can be measured in the negative mode, which is more sensitive as it produces less background noise (Harnly et al. 2007). Additionally, the negative mode gives fragments that enable anthocyanins to be differentiated from other flavonoid glycosides (Sun et al. 2012). However, most authors have used positive ionization for anthocyanins (Ieri et al. 2012), and negative as well as positive modes to investigate phenolic compounds to gain additional information from minor fragments for the identification of compounds especially in combination with fragmentation (MS/MS or MS^n). The main characteristic fragment ions of flavonoids are found irrespective of the ionization technique and the mass spectrometer used (de Rijke et al. 2006). Rauha et al. (2001) found ESI ionization in the negative mode to have the highest sensitivity. Acidification of the samples with formic acid or acetic acid during the HPLC-run may influence the efficiency of ionization (better protonation in positive mode) and seldom causes adducts with the flavonoids. Different kinds of mass spectrometers have been used to identify the flavonoid profile of plants. TOF mass spectrometers can measure in high resolution mode and therefore give the exact mass (Cheng et al. 2011). They are therefore often used in the analyses of phenolic compounds in specially targeted experiments. The formula of a compound can be calculated, and quantification may also be done with this kind of mass spectrometer (Cheng et al. 2011). However, to identify a compound, additional chromatographic data are needed such as absorption maxima, retention time and elution

order. Ion trap mass spectrometers give additional information on fragmentation patterns after collision-induced dissociation (CID) of molecule ions, and can help to identify different structures by subsequent fragmentation, usually up to MS^5 providing a lot of information about the unknown compound (Schmidt et al. 2010), but fail to work at high resolution. Triple quadrupol mass spectrometers are mainly used for quantification, but additionally allow MS/MS experiments that are used for targeted analysis (Chen et al. 2012). Currently, quadrupol, TOF and ion trap techniques are combined to provide structural information by MS/MS or MS^n, giving high resolution as well as reliable quantification at the same time. Further new techniques that combine fragmentation and high resolution are also being developed (e.g. Thermo Scientific-ORBITRAP).

In-source fragmentation of phenolics and CID reveal much information about the structures of flavonol aglycones, flavonol glycosides and other phenolics. For flavonoid aglycones, fragmentation breaks the C-C linkages of the C-ring in a retro-Diels-Adler reaction and therefore leads to typical fragments (Justesen 2000, Fabre et al. 2001, de Rijke et al. 2006). To identify unknown phenolics it is nessessary to identify the aglycons after acid hydrolysis (e.g. Slimestad et al. 2007, Fiol et al. 2012, Tatsuzawa et al. 2012). However, different functional groups in the flavonoid aglycone can produce differences in fragmentation e.g. a methoxy group as in isorhamnetin is characterized by the loss of 15 u (units) (de Rijke et al. 2006). The specific fragment ions of flavonoid glycosides allow the glycosides linked to the flavonoid aglycone as well as the interglycosidic linkages to be indentified (Cuyckens et al. 2003, de Rijke et al. 2006**).** For instance, 71 kale flavonol glycosides including partly acylated mono- to penta-glycosides have been identified by the fragmentation patterns up to MS^5 (Fig. 3) (Schmidt et al. 2010). As well as identifying the kind of sugar moiety 162 u (hexose), 146 u (deoxyhexose), 132 u (pentose) or 176 u (uronic acid), information on the interglycosidic linkages can be obtained by MS (de Rijke et al. 2006). Ferreres et al. (2004) reported that both sophorosides (1→2 glycosidic linkage) and gentiobiosides (1→6 glycosidic linkage) are characterized by the loss of 324 u. In addition, flavonoid sophorosides were defined by the fragment ion $[M\text{-}H\text{-}180]^-$ and were also able to produce the fragment ions $[M\text{-}H\text{-}162]^-$ and $[M\text{-}H\text{-}120]^-$ (Ferreres et al. 2004). In contrast to flavonoid O-glycosides, in flavonoid *C*-glycosides the sugar is linked to the C-6 or C-8 position of the flavonoid molecule via an acid-resistant C-C bond. The fragmentation of flavonoid *C*-glycosides is characterized by cleavage of the glucose moiety and loss of water molecules (Waridel et al. 2001, de Rijke et al. 2006). Due to the lack of standards for many flavonoid glycosides, and other phenolic compounds, mass spectrometry can often only provide tentative identification and NMR spectra are needed for

structural elucidation.

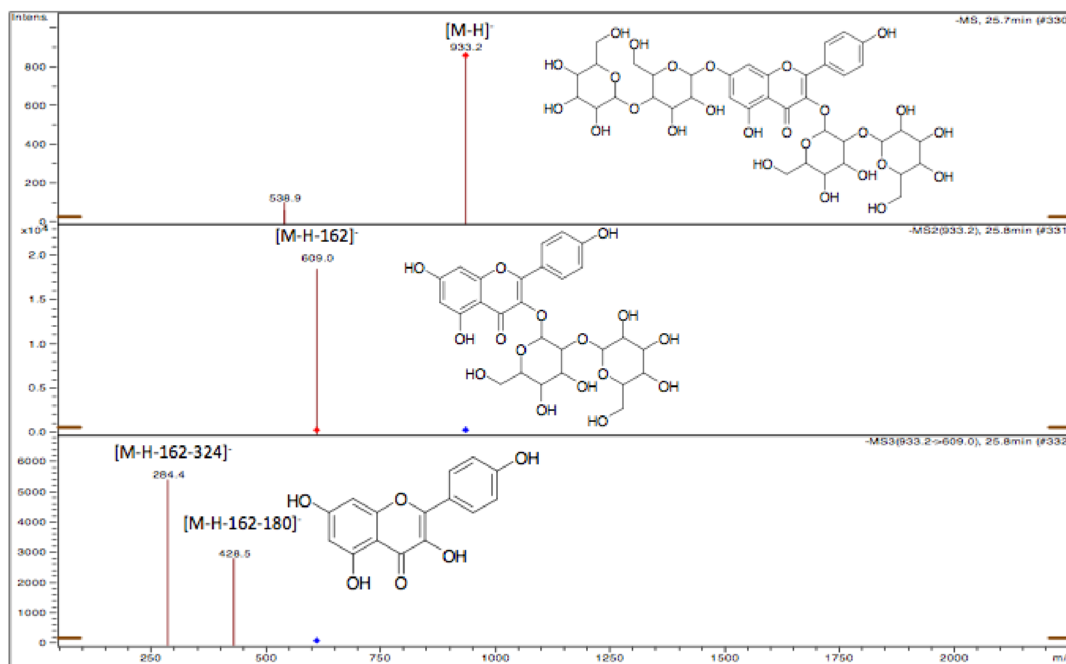

Fig. 3. MS-analysis of kaempferol 3-*O*-sophoroside 7-*O*-diglycoside.

Quantitative and qualitative NMR analysis of flavonoids

Structural identification of flavonoid glycosides is essential for gaining an understanding of their function in the plant and their nutritional value for humans (e.g. Jwanny et al. 2012). Nuclear Magnetic Resonance (NMR) is the best method for identifying and quantifying unknown organic compounds, even when these are part of complex mixtures. Traditionally, concentrations of studied compounds had to be present in mg/ml (mM) for analysis. Nowadays, thanks to improved magnetic strengths (up to 1 GHz, high field NMR spectrometers) and novel cryoprobe techniques, compounds present in µg/ml (µM) concentrations can be accurately characterized. Moreover, NMR techniques can also be used for non-target analyses of plant extracts to pinpoint characteristic changes in the metabolomic profile caused by, for example, different treatments, or origins of plants (Ignat et al. 2011), or for mapping out

total concentrations of different classes of organic molecules, like aldehydes, aromatic compounds, carbohydrates, organic acids and amino acids e.g. from *Allium* spp. juice (Soininen et al. 2012).

Prior to NMR measurements, liquid or solid samples need to be dissolved into deuterated solvents. Methanol (i.e. CD_3OD) is the main deuterated solvent used for flavonoid identification, and although dimethylsulfoxide (DMSO-d_6) is also used (Cao et al. 2011) its high boiling point (189 °C) compared to CD_3OD is problematic. Yet, the advantage of DMSO is that it enables detection of chemical shifts and coupling constants for the exchangeable (e.g. OH) protons, making for easier structure assignment of polyphenols. Typically, tetramethylsilane (TMS) is used as an internal chemical shift standard, but it is preferable to use a known concentration of non-volatile trimethylsilyl propanoic acid sodium salt (TSP), since this allows simultaneous accurate assessment of chemical shifts and quantification of known compounds (Soininen et al. 2012). However, a drawback of using TSP is that it binds to proteins, making quantification less accurate when the protein concentration is high (>2%). During the NMR measurements samples remain unchanged, since only a couple of mJs of energy are absorbed by the sample.

The quantification of a known compound present in a mixture is easy if at least one of the chemical shifts from that compound can be clearly distinguished from all other signals. However, the situation is more complicated when chemical shifts overlap and/or the baseline is distorted by spectral artifacts or background proteins. Recently, a convenient NMR method for metabolite profiling in plants has been developed based on a constrained total-line-shape (CTLS) approach (Soininen et al. 2012). The CTLS approach helps to minimize problems arising from signal overlap, chemical shift variations and spectral artifacts, including background proteins. At present, there are only a few quantitative NMR studies of flavonoids (e.g. Pacifico et al. 2011). This limitation is expected to be lifted in the near future as cryoprobe techniques in high field instruments become more widespread.

To identify the different classes of flavonoids that are present in plant extracts, one-dimensional NMR techniques with ^{1}H and ^{13}C NMR can be applied (Disadee et al. 2011,Xü et al. 2011, Fiol et al. 2012, Qu et al. 2012, Tatsuzawa et al. 2012). Common 2D NMR techniques are also sometimes used for flavonoid analysis (Disadee et al. 2011, Xü et al. 2011, Fiol et al. 2012, Qu et al. 2012, Tatsuzawa et al. 2012). The most common 2D NMR techniques are: ^{1}H-^{1}H COSY (correlation spectroscopy), which solve out protons next to each other in the structure; ^{1}H-^{13}C HSQC (heteronuclear single quantum coherence) which detects direct one bond correlations between ^{1}H and ^{13}C NMR chemical shifts (no correlation is observed for heteroatom bound protons), and ^{1}H-^{13}C HMBC (heteronuclear

multiple bond correlation) which maps out the 2-4 bond correlations between ^{1}H and ^{13}C NMR chemical shifts, including the correlation with heteroatom bound protons (e.g. O-H). In the case of complex flavonoid glycosides, acid hydrolysis can help to identify components of the molecule such as the aglycone or the sugar moieties (Fiol et al. 2012, Tatsuzawa et al. 2012, Formisano et al. 2012, Slimestad et al. 2007).

The most common procedure for compound identification, comprised of HPLC purification followed by separate NMR identification, is rather laborious and time-consuming. While the first LC-MS instrument was developed ca. 40 years ago, the first HPLC-NMR systems were introduced less than 20 years ago (Albert 1995), followed by HPLC-NMR-MS instruments (Lindon et al. 2000); but these NMR-based techniques lack the sensitivity required for flavonoid studies. Subsequently, to attain the necessary sensitivity, an individual on-line solid phase extraction (SPE) cartridge has been added after the HPLC column, in which the (UV-absorbing) peaks of interest are trapped (Xu et al. 2011). This allowed transfer of concentrated analytes, through capillaries, directly into a special small-volume (e.g. 30 μl) NMR flow cell for 1D and/or 2D NMR measurements. Recently, improved hyphenated LC/DAD/SPE/NMR and LC/UV/ESI-MS systems have been used for separation and structure verification of several flavonoids that are present in *Hypericum perforatum* extracts (Tatsis et al. 2007). The latest invention is an automated MS-guided LC-MS-SPE-NMR instrument, in which flavonoids from tomato extracts have been selected based on MS data (Hooft et al. 2011). After trapping and drying the specific compounds on individual SPEs, the analytes were transferred to a NMR spectrometer and the required spectra were measured. Compounds were identified based on accurate MS and MS^{n} spectral data, information from NMR measurements and a database were used to predict ^{1}H NMR chemical shifts.

In addition to liquid state NMR measurements, flavonoid samples have also been studied using solid state (SS) NMR. SS NMR spectra for flavonoids are less useful than liquid NMR, since spectral lines are broad, typically requiring at least 10-100 mg of a compound. However, this method has been successfully applied to study tannin structures in *Acacia mangium* (Willd.) (Hoong et al. 2010) to map out intramolecular hydrogen bonds in solid flavonoids, like morin and kaempferol (Zielińska et al. 2008), and to characterize the structures of baicalein, baicalin and wogonoside (Wolniak et al. 2007).

Non-destructive methods to assess flavonoid content in situ

The flavonoid content of leaves and other plant parts can be assessed using non-destructive methods that are based on their optical properties in the UV and visible spectral regions. Chlorophyll (Chl) molecules excited by both UV and visible radiation emit fluorescence that may then be detected at appropriate wavelengths. Differences in chlorophyll fluorescence (ChlF) caused by excitation in the UV ($ChlF_{UV}$) and the visible bands ($ChlF_{VIS}$) are used to assess the amount of UV-absorbing compounds located in the epidermis (Figure 4). The $ChlF_{UV}$ / $ChlF_{VIS}$ ratio represents the UV transmittance (i.e. lack of absorbance) of the epidermis. The basics of the ChlF excitation screening (ChlFES) method and its limitations are given in Bilger et al. (2001), Cerovic et al. (2002), Agati et al. (2005) and Agati et al. (2007). The ChlFES technique allows continuous assessment of changes in UV-absorbing compounds accumulation over time in one particular sample. Therefore, this is a powerful tool for comparative studies and high throughput screening. The limitations of this method are that only epidermal compounds are detected and that it cannot give information on the flavonoid composition.

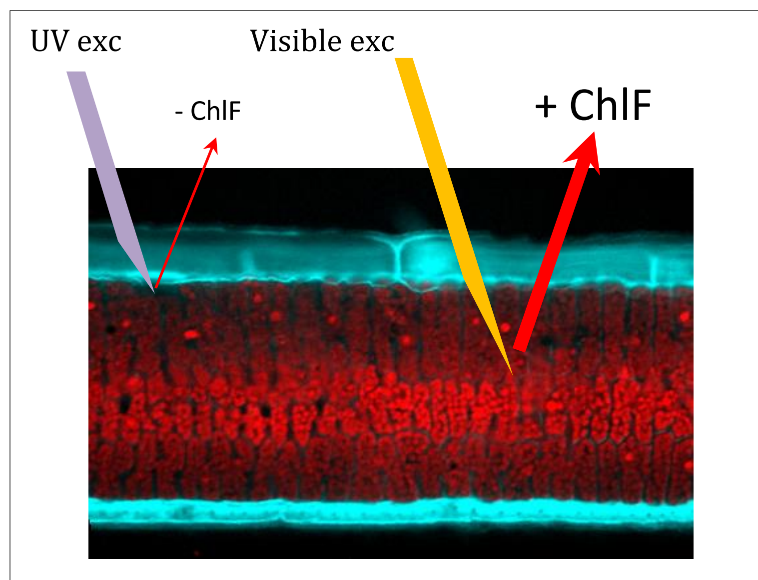

Fig. 4. Schematic representation of the chlorophyll fluorescence excitation screening (ChlFES) method using a wheat leaf longitudinal section (autofluorescence microimage under 365 nm excitation of chlorophyll (red) and phenolic compounds (light blue). UV excitation is attenuated by phenolic compounds in the epidermis resulting in less chlorophyll fluorescence (-ChlF). Visible excitation fully penetrates to mesophyll resulting in higher chlorophyll fluorescence (+ChlF). The thickness of the arrows indicates relative intensity. Comparison of the two ChlF signals provides *in vivo* quantification of the epidermis transmittance.

The ChlFES technique was first applied by the Xe-PAM fluorometer (Walz, Effeltrich, Germany) (Bilger et al. 1997). This system is not truly non-invasive, as it requires dark-adapted leaf discs. The system produces excitation in the UV-B (λ_{max} 314 nm), UV-A (λ_{max} 366 nm) and blue-green (400–550 nm, as a reference signal) parts of the spectrum, and has been used to investigate the UV-B-induced accumulation of flavonoids in the epidermis (Nybakken et al. 2004a and 2004b, Kolb at al., 2005, Kolb and Pfündel 2005, Bilger et al., 2007). A positive linear relationship has been reported between the $ChlF_{UV}$ / $ChlF_{VIS}$ ratio and the transmittance of freshly peeled leaf epidermal tissue measured using an integrating sphere (Barnes et al. 2000) or using a spectrophotometer with a turbid sample setup (Markstadter et al. 2001). Burchard et al. (2000) obtained a good positive correlation between the epidermal UV-A and UV-B absorbance of *Secale cereale* leaves and the epidermal flavonoid content determined by HPLC analysis. In *Vicia faba*, epidermal absorbance determined using fluorescence measurements was also positively correlated with the absorbance at 366 nm and 314 nm of methanolic extracts (Bilger et al. 2007). Curvilinear relationships between epidermal absorbance, determined using fluorescence measurements, and total phenolic UV absorbance in extracts have been reported for *Vitis* spp. and *Hordeum vulgare* leaves (Kolb and Pfündel 2005). Thus, relationships between the measured $ChlF_{UV}$ / $ChlF_{VIS}$ ratio and phenolic content are, to some extent, species specific, and this may reflect species-dependent differences in the location of UV-screening pigments.

A portable UV-A PAM fluorometer (Gademann Instruments, Würzburg, Germany) device has also been used to measure epidermal UV transmittance of leaves *in-situ* (Bilger et al. 2001). Light emitting diodes (LEDs) produce excitation radiation in the UV-A (peak 375 nm) and blue (peak 400 nm) wavelength regions. ChlF is measured at wavelengths above 650 nm. The instrument allows assessment of UV-screening compounds only in the UV-A band (at 375 nm), although many flavonoids have absorption peaks at shorter wavelengths, typically in the UV-B region. ChlFES spectroscopy studies have shown that detection of flavonoids using an excitation wavelength centred at their absorption maximum can be missleading at high concentrations of the compounds (Cerovic et al. 2002; Bidel et al. 2007; Agati et al. 2011). Then, by using 375 nm radiation (i.e. the tail of flavonoid absorption) for excitation, detection over a larger range of concentrations is possible. Epidermal absorbance measured using a UV-A PAM was found to correlate well with the quercetin content of *Brassica oleracea* leaves, determined by HPLC (Hagen et al. 2009). Kolb et al. (2005) compared UV-B transmittance, measured using a Xe-PAM system, with UV-A transmittance measured using a UV-A PAM fluorometer, for *Vitis* spp. and *H. vulgare* leaves. The authors concluded that a good assessment

of compounds absorbing in the UV-B band is possible with the UV-A PAM fluorometer once the relationship between the absorbance in the two UV bands has been defined.

The use of blue-excited ChlF as a reference signal by the Xe-PAM and UV-A PAM renders them inapplicable for use with anthocyanin containing leaves (Barnes et al. 2000; Pfündel et al. 2007), *Malus* spp. (red Aroma variety) (Hagen et al. 2006), or any other tissue containing blue-absorbing compounds. This problem is circumvented with the Dualex portable instrument (Force-A, Orsay, France: Goulas et al. 2004), which uses excitation bands in the UV-A (λ_{max} 375 nm) and in the red (λ_{max} 657 nm) rather than in the blue band. ChlF is detected in the near-infrared, at about 730 nm. This instrument facilitates use of leaf discs and small leaves, which are positioned between the excitation head and the detection head. Thick leaves (> 1 mm) may produce questionable readings since the excitation LEDs and fluorescence detector are placed on opposite sides of the leaf. Positive correlations have been found between Dualex epidermal absorbance (the sum of absorbance of both leaf sides) and total flavonoid content obtained using HPLC in *Vitis* spp. leaves but there is no correlation with hydroxycinnamic acid contents (Agati et al., 2008a). Dualex (series 4) instruments have been employed to detect epidermal UV transmittance in dicots and monocots (Louis et al., 2009; Morales et al., 2010, Cerovic et al., 2012), since both flavones and flavonols are among the major phenolic compounds absorbing at 375 nm. A comparison of the UV-A-PAM and Dualex, using leaves from several species, yielded similar absorbance values (Pfündel et al., 2007). In a further methodological study, epidermal UV attenuation of *Fraxinus excelsior* (L.) and *Acer platanoides* (L.) was measured using the Dualex fluorometer, spectrophotometry of extracts, and the Folin-Ciocalteu colorimetric method. Close correlations were found between Dualex and spectrophotometric readings, but all correlations were highly species-specific (Barthod et al., 2007).

The development of the Multiplex sensor (Force-A, Orsay, France) has allowed for detection of flavonoids over a large sample area, up to 8 cm in diameter. The sensor uses one UV source (375 nm) and three LED-matrices emitting at 470 nm, 516 nm and 635 nm (Ben Ghozlen et al. 2010). The Multiplex has three detection channels in the blue-green, red and far-red spectral regions: the latter two detecting 680–690 nm and 730–780 nm fluorescence, respectively, corresponding to the two emission peaks of chlorophyll. As the LED sources are pulsed and synchronized for detection, the sensor is insensitive to ambient light. The Multiplex sensor has been used to assess the flavonol content of *Malus* spp. (Betemps et al. 2012) and kiwifruit exocarps of *Actinidia deliciosa* (Pinelli et al. 2013), as well as bunches of *Vitis vinifera* (Cerovic et al. 2008). Using the Multiplex sensor, a flavonoid index was also

measured for the apical part of *Ligustrum vulgare* (L.) plants cultivated at 30% or 85% of full sunlight in the presence, and in the absence, of UV radiation. The flavonoid index was higher in leaves grown at 85% than at 30% sunlight, irrespective of UV irradiance (Agati et al. 2011). Transferring plants, acclimated for 3 weeks to 30% sunlight, to 85% sunlight, caused an exponential increase in the flavonoid index, which reached a maximum within 10 days (Agati et al. 2011). These data demonstrate the value of ChlFES methods in non-invasive monitoring of acclimation dynamics.

The ChlFES method can also be applied as an imaging technique to detect the spatial distribution of UV-absorbing compounds in the leaf lamina (Mazza et al. 2000). Multi-spectral fluorescence imaging has been used to obtain the distribution of UV-shielding compounds in *Fagus sylvatica* L. leaves. Within a leaf, the highest UV-shielding was found at the leaf rim (Lenk and Buschmann 2006). Likewise the adaxial epidermis possessed more UV-shielding pigments than the abaxial epidermis. ChlF excitation ratio imaging has also been used to visualize *in vivo* flavonols within white berries of *Vitis vinifera* (Lenk et al. 2007), and anthocyanins within whole bunches of red *Vitis vinifera* (Agati et al. 2008b). Application of the imaging technique at a microscopic scale has allowed mapping of the epidermal UV-absorbance in immature and mature leaves of *Quercus petraea* (Meyer et al. 2009).

Conclusions

The enormous amount of published literature on flavonoid analysis and the inter-comparison of data indicate that it is difficult to recommend a single, straightforward analytical procedure. Many factors need to be considered, including target compounds, matrix examined and the cost. Sample collection and pre-treatment of plant material are of pivotal importance so that the results are representative of fresh, intact material. Freeze-drying, immediately after sample collection, minimises loss of target compounds. We note a trend towards fast extraction techniques or the combination of hyphenated techniques with liquid chromatographic systems, as a means to reduce the cost of analysis and optimise the quality of the information obtained. Nevertheless, knowledge of the stability of specific target compounds and the use of response surface methodology will help the selection of the most appropriate conditions for extraction.

Chromogenic assays can provide useful information on the flavonoid content of tested matrices. However, the term "total flavonoid content" should be avoided as there is no accepted and universal

method for the measurement of total flavonoid. Moreover, many chromogenic assays need to be validated as they show variable degrees of specificity for different flavonoid aglycones and little is known about their glycosides. Due to its simplicity and versatility, TLC upgraded to the high performance version is expected to continue as the "workhorse" for flavonoid analysis. At the moment, HPLC and combinations of UHPLC with mass spectrometry and of LC and NMR are still the most powerful, though also the fairly expensive tools for studies of flavonoids under UV-B conditions.

Finally, ChlFES methods for the non-destructive assessment of epidermal UV-screening flavonoids have now been validated in many studies. This method cannot substitute for the analytical chemistry of purified individual flavonoids. Yet, the flexibility and potential to perform a large number of parallel measurements with minimal sample preparation repeated on the same target over a period of time, makes ChlFES an important tool for studies of UV response of flavonoids.

In conclusion, many different tools are available to study the accumulation and functional role of flavonoids in plants. Every analytical tool has advantages and disadvantages, and consequently there is no single method that can be applied to every single flavonoid study. Plants have evolved a bewildering array of flavonoid-metabolites for specific biological functions; while researchers have responded by developing an ingenious assortment of analytical techniques for specific purposes. The message of this review is that researchers will need to carefully consider the biological process that they intend to study, and choose an analytical method that best addresses their specific objectives.

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