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SPECIAL FEATURE: TUTORIAL

Mass spectrometry in the structural analysis of flavonoids

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Flavonoids are very common and widespread secondary plant metabolites. They have a wide range of biological and physiological activities and serve as chemotaxonomic marker compounds. Therefore, they have been extensively investigated both in the past and during recent years. The interest in them is still increasing. In the search for new compounds, and also in quality control, there is a need to have reliable methodology for the analysis of flavonoids. Mass spectrometry can make an invaluable contribution because of its high sensitivity, possibilities of coupling with liquid chromatography and the availability of powerful tandem mass spectrometric techniques. A review of currently available mass spectrometric methodology used in the structure elucidation of flavonoids is presented. Sample preparation, liquid chromatographic/mass spectrometric analysis and tandem mass spectrometric procedures for the characterization of flavonoid aglycones, *O*-glycosides, *C*-glycosides and acylated glycosides are considered. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: liquid chromatography/mass spectrometry; tandem mass spectrometry; collision-induced dissociation; flavonoid glycosides; electrospray ionization

INTRODUCTION

General considerations

It is estimated that about 2% of all carbon photosynthesized by plants, amounting to about 1×10^9 tonnes per year, is converted into flavonoids or related compounds.[1] With very few exceptions, only plants possess the ability to biosynthesize flavonoids, in contrast to animals and fungi.[2] Flavonoids occur in all parts of plants. Flavonoid aglycones are found in farinose exudates or wax on leaves, barks and buds, or as crystals in cells of cacti. In most cases, flavonoids are present as glycosides in vacuoles of flowers, leaves, stems or roots. Flavonoids comprise the colored pigments of flowers, but also act as enzyme inhibitors, as a defence system against ultraviolet radiation exposure and insects, and as chelating agents of metals that are noxious to plants. They are also involved in photosensitization and energy transfer, morphogenesis and sex determination, photosynthesis and regulation of plant growth hormones.[3] Because there is a strong tendency for chemotaxonomically related plants to produce similar types of flavonoids, they are commonly used as chemotaxonomic markers.[2] Most interest, however, is in their physiological actions and their beneficial effects on human health. The best documented property of almost all groups of flavonoids is their ability to act as antioxidants.

Flavonoids can interfere with at least three different free radical-producing systems.[4] Owing to their lower redox potentials, they are able to reduce highly oxidizing free radicals[5] by forming less reactive flavonoid radicals and so prevent for example lipid peroxidation,[6] one of the most important actions of free radicals that leads to cellular membrane damage and, ultimately, to cell death. Flavonoids are also able to scavenge nitric oxide[7] which forms in combination with superoxide free radicals the highly damaging peroxynitrite, and also to inhibit xanthine oxidase,[8] an important biological source of superoxide radicals. Superoxide radicals can react with hydrogen peroxide, which gives rise to the even more toxic hydroxyl radicals. This reaction, called the Fenton reaction, is catalyzed by iron and can be inhibited by quercetin, which has iron-chelating effects.[9] Flavonoids are used in the prevention of cancer,[10] dementia,[11] atherosclerosis[12] and coronary heart disease.[13,14] They also interact with various enzymatic systems, e.g. their inhibition of the enzymes cyclooxygenase and lipoxygenase.[15,16] This results in a decrease in platelet activation and aggregation that contributes to their protection

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against cardiovascular diseases and also to their anti-inflammatory activity.[17] Many other biological activities are attributed to flavonoids, e.g. antiviral, antimicrobial, antihepatotoxic, antiosteoporotic, antiallergic, antispasmodic and antiulcer activity. Several reviews concerning their mechanisms of action and their potential therapeutic uses have been published recently.[3,4,18,19]

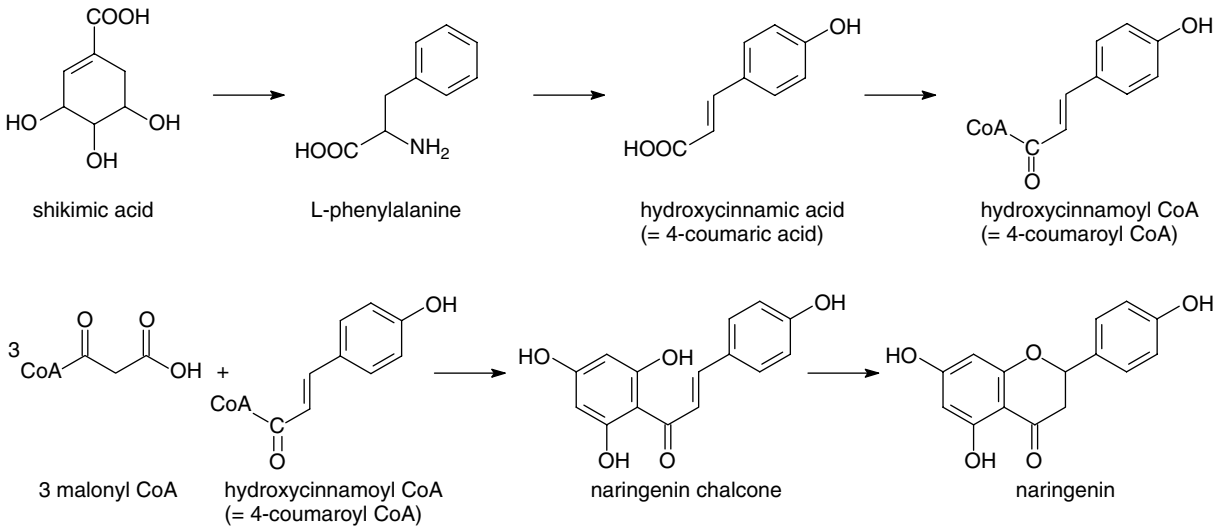
Data on absorption, metabolism and excretion of dietary flavonoids are scarce and sometimes contradictory, but very relevant to their beneficial effects on human health. Whether flavonoid glycosides[18,19] or only aglycones[20] are able to pass the gut wall continues to be a subject of dispute. Deglycosylation seems to be the most likely first step during transfer of flavonoids into the circulation, consistent with the strong β-glycosidase activity within the epithelial cells of the small intestine.[21,22] After absorption, flavonoids are extensively metabolized, namely, hydroxyl groups are conjugated with glucuronic acid, a sulfate or a methyl group.[23,24] In the colon, microorganisms can degrade unabsorbed flavonoids and conjugated flavonoids excreted in bile, explaining the large inter-individual variations in bioavailability owing to the different composition of the intestinal microflora.[25] The intake of flavonoids varies greatly between countries,[4,23] depending on the habitual diet. The main flavonoid dietary sources are fruits and beverages (fruit juice, red wine, tea, coffee and beer) and, to a lesser extent, herbs and vegetables.[25–27] Owing to the widespread occurrence in edible plants in the human diet and the health-related properties that are attributed to flavonoids, they have been subject to ever-increasing attention.

For the investigation of structure–activity relationships and food quality control, it is important to have access to rapid and reliable methods for the analysis and identification of these natural polyphenolic compounds in all their many forms. Modern mass spectrometric techniques are very well suited to the analysis of flavonoids in plants and foodstuffs and play a key role in the analysis, since they can provide significant structural information on small quantities of pure samples as well as on mixtures. The different mass spectrometric techniques that can be applied to analysis of flavonoid glycosides have been reviewed by Stobiecki.[28] Liquid chromatography coupled to mass spectrometry (LC/MS) represents a very powerful tool for the analysis of natural products. The mass spectrometer is a universal detector that can achieve very high sensitivity and provide information on the molecular mass and on structural features. More detailed structural information can subsequently be obtained by resorting to tandem mass spectrometry (MS/MS) in combination with collision-induced dissociation (CID). With regard to structure characterization of flavonoids, information can be obtained on (1) the aglycone moiety, (2) the types of carbohydrates (mono-, di-, tri- or tetrasaccharides and hexoses, deoxyhexoses or pentoses) or other substituents present, (3) the stereochemical assignment of terminal monosaccharide units, (4) the sequence of the glycan part, (5) interglycosidic linkages and (6) attachment points of the substituents to the aglycone. In this paper, the currently available mass spectrometric methodology for obtaining structural information on flavonoids is reviewed.

Chemical structure

Flavonoids are formed by a series of condensation reactions between hydroxycinnamic acid (B-ring and carbon atoms 2, 3 and 4 of the C-ring) and malonyl residues (A-ring), giving rise to a $C_6—C_3—C_6$ base structure (Scheme 1). The three-carbon bridge between the phenyl rings is commonly cyclized to form a third ring (C-ring).

According to the cyclization and the degree of unsaturation and oxidation of the three-carbon segment, they can be classified into several groups. The basic structures of the main classes of flavonoids are shown in Fig. 1. At present, about 400 flavone aglycones, 450 flavonol aglycones, 350 flavanone aglycones, 300 isoflavone aglycones, 19 anthocyanidins and 250 chalcone aglycones have been reported.[2] In plants, flavonoids may occur in various modified forms corresponding to additional hydroxylation, methylation and, most importantly, glycosylation. Occasionally, aromatic and aliphatic acids, sulfate, prenyl, methylenedioxyl or isoprenyl groups also attach to the flavonoid nucleus and their



Scheme 1. Biosynthesis of flavonoids.

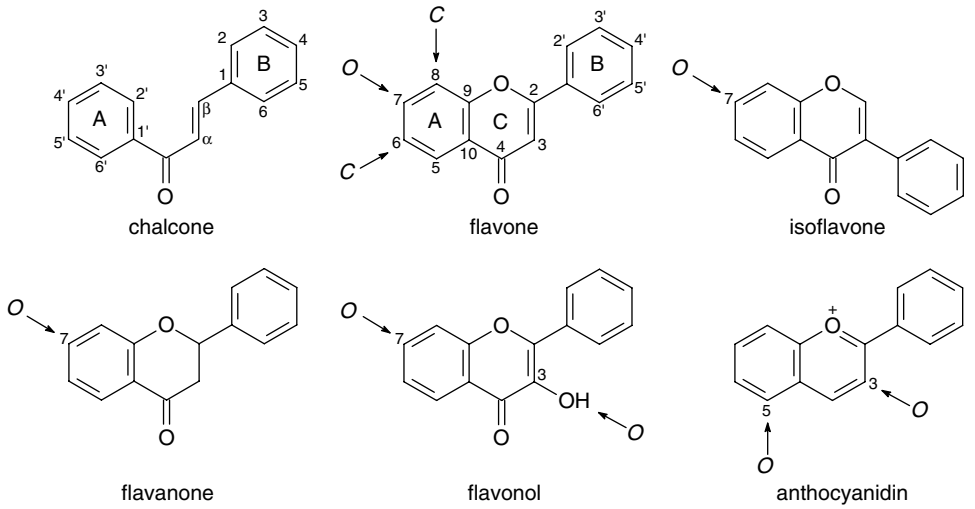


Figure 1. Basic structures of the main classes of flavonoids. Common *O*- and *C*-glycosylation positions are indicated with an arrow.

glycosides.[2] Flavonoids commonly occur as flavonoid *O*-glycosides, in which one or more hydroxyl groups of the aglycone are bound to a sugar with formation of a glycosidic O—C bond, which is an acid-labile hemiacetal bond. The effect of glycosylation is to render the flavonoid less reactive and more water soluble, so that glycosylation can be regarded as an essential form of protection in plants to prevent cytoplasmic damage and to store the flavonoids safely in the cell vacuole.[29] In principle, any of the hydroxyl groups can be glycosylated but certain positions are favored: for example, the 7-hydroxyl group in flavones, flavanones and isoflavones, the 3- and 7-hydroxyls in flavonols and flavanols and the 3- and 5-hydroxyls in anthocyanidins are common glycosylation sites (Fig. 1). 5-*O*-Glycosides are rare for compounds with a carbonyl group on position 4, since the 5-hydroxyl group participates in hydrogen bonding with the adjacent 4-carbonyl group. Glucose is the most commonly encountered sugar, galactose, rhamnose, xylose and arabinose are not uncommon, and mannose, fructose, glucuronic and galacturonic acids are rare.[2,30] Disaccharides are also often found in association with flavonoids, the more common ones being rutinose (rhamnosyl-(α1 $\rightarrow$ 6)-glucose) and neohesperidose (rhamnosyl-(α1 $\rightarrow$ 2)-glucose) and occasionally tri- and even tetrasaccharides. Acylated glycosides, in which one or more of the sugar hydroxyls are esterified with an acid, also occur. Glycosylation may also take place by direct linkage of the sugar to the flavonoid's basic nucleus, via an acid-resistant C—C bond, to form flavonoid *C*-glycosides. Flavonoid *C*-glycosides are commonly further divided into mono-*C*-glycosylflavonoids, di-*C*-glycosylflavonoids and *C*-glycosylflavonoid-*O*-glycosides.[31] In the last category, a hydrolyzable sugar is linked either to a phenolic hydroxyl group or a hydroxyl group of the *C*-glycosyl residue. To date, *C*-glycosylation has only been found at the C-6 and/or C-8 position of the flavonoid nucleus (Fig. 1).

SAMPLE PREPARATION

The need for sample preparation depends strongly on the sample type and the analysis techniques used. Various procedures for sample preparation and clean-up of flavonoid samples can be used, as recently reviewed by Robards and co-workers.[32,33] The procedures must allow quantitative recovery of flavonoids, whilst avoiding any chemical modification or degradation. Owing to the high selectivity of mass spectrometry, especially in combination with LC or MS/MS, no extensive sample preparation is required. Flavonoid samples can be prepared by homogenization, liquid extraction and filtration and/or centrifugation. The extraction conditions, i.e. temperature, pH and extraction solvent, can have a considerable influence on the kind and amount of flavonoids isolated.[34] Sometimes additional processing is desirable, e.g. to purify and enrich a certain flavonoid or flavonoid fraction, to discard any interfering matrix components or to eliminate highly lipophilic compounds that can adsorb on reversed-phase (RP) LC columns. To date, solid-phase extraction (SPE) is the method of choice.[32,35] The use of mini-cartridges, usually filled with C_{18} RP material, allows a simple and rapid purification and preconcentration of flavonoids, where recoveries are comparable to those with simple filtration.[36] The sample solution and eluents are preferentially acidified in order to suppress the ionization of the flavonoids and as such to increase their retention.[37] An identical procedure can be used as a successful desalting method.[38]

Newer sample preparation techniques, e.g., supercritical fluid extraction (SFE),[39,40] pressurized liquid extraction (PLE)[41,42] and solid-phase microextraction (SPME),[43] have also found their way into flavonoid analysis. They are more easily automated, and solvent consumption and analysis times are reduced, resulting in higher throughput and minimizing the possibility of alteration and degradation of the sample compounds. More detailed information concerning these and other new sample preparation techniques can be found in recent reviews.[43–47]

LIQUID CHROMATOGRAPHIC/MASS SPECTROMETRIC ANALYSIS

Flavonoids are generally part of a complex mixture isolated from a plant extract, making a purification step necessary for adequate analysis. Also, the amount of plant extract

is often limited, so that on-line techniques are to be preferred, which have the additional advantage in that they also provide faster analysis. Gas chromatography (GC)/MS is not widely used in flavonoid analysis owing to the limited volatility of flavonoid glycosides. Derivatization is needed, making the analysis more time consuming, and the fragmentation patterns of the derivatives are often difficult to interpret. Since the development of atmospheric pressure ionization (API) sources, LC/MS coupling became more efficient and easy to use, making it by far the most popular technique for on-line flavonoid analysis nowadays. Also, the coupling of capillary electrophoresis (CE) with MS is possible. Although CE allows a higher separation efficiency than LC and may be a valid alternative technique,[48,49] CE/MS has found only very limited use in flavonoid analysis.

LC/MS is rarely used for full structure characterization, but it provides the molecular mass of the different constituents. With this knowledge, LC/MS/MS and MS/MS analysis can be pursued for further structure characterization. Additionally, it can be used to determine the occurrence of previously identified compounds, and so minimizes the effort lost in their isolation.[50] It is also employed for quantitative analysis or is suited to the identification of labile compounds in solution, such as acylated flavonoids. The efficiency of different API sources, i.e., electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI), has been compared by Rauha *et al*.[51] The highest sensitivity is obtained using ESI in the negative ion mode with an eluent system consisting of an acidic ammonium acetate buffer. Both APCI[52–54] and ESI[34,51,55] appear to be favored by different authors for the analysis of flavonoids. In all these studies, the elution conditions are found to be critical. The eluent system usually consists of an acidified aqueous solvent and acetonitrile or methanol. Overviews of LC methods used in flavonoid analysis in the last decade have been given by Merken and Beecher[35] and Robards and Antolovich.[56] Acidification provides a better retention and separation on the C_8- and C_{18}-RP columns which are almost exclusively employed. Formic, acetic and trifluoroacetic acid and ammonium acetate and formate are volatile and thus compatible with LC/MS systems. Comparative studies show that the lowest detection limits in the positive ion mode are obtained with an overall concentration of around 0.5% formic acid.[51,57] Trifluoroacetic acid decreases the sensitivity owing to its strong ion-pairing effect,[58] making the analyte ions appear like neutrals. The opposite holds for acetic acid, which has weak ion-pairing capacity. Acetic acid slightly increases the ESI efficiency but at the same time it also strongly reduces the retention on the LC column. To compensate for this effect, the amount of organic solvent in the mobile phase has to be reduced, which in turn decreases the sensitivity.[57] A similar decrease in retention is noted for isoflavone 6″-*O*-malonylglucoside conjugates owing to ionization of the free carboxyl group when an ammonium acetate buffer is used.[52] In the negative ion mode, a concentration of 0.1% formic acid[57] or 5 mM ammonium acetate buffer[51] seems to be preferable, whilst the addition of basic eluents has a negative influence on both the LC separation and the ESI process. The negative ion mode provides the highest sensitivity[54,55,57,59–63] and results in limited fragmentation, making it most suited to infer the molecular mass of the separated flavonoids, especially in cases where concentrations are low. The peak at the highest m/z ratio is not always the molecular ion species ($[M + H]^+$ in the positive mode, and $[M - H]^-$ in the negative mode), because adducts with solvent and/or acid molecules[52,54] and also molecular complexes ($[2M + H^+$ or $[2M - H]^-$)[64] can be generated. An increase in cone voltage reduces the incidence of both adduct and complex formation.[52,55] The first-order mass spectrum in the positive ion mode contains more structural information than that in the negative ion mode, which is useful to identify known compounds, whereas the combined use of both ionization modes gives extra certainty to the molecular mass determination, especially for the minor compounds where the noise level is much higher.

Structural information can also be obtained from the chromatographic retention times. For the C_{18}- or C_8-RP columns generally used, the more polar compounds are eluted first. Thus, retention times are inversely correlated with increasing glycosylation, whereas acylation, methylation or prenylation have the opposite effect, although the position of glycosylation[65] or methylation[66] can have a significant effect on the retention time. Flavanones precede flavonols, which in turn precede flavones for compounds with an equivalent substitution pattern. For isomeric compounds that differ in the structure of the saccharide residues, rutinosides elute ahead of neohesperidosides, galactosides ahead of glucosides,[56] glucosides ahead of arabinosides and arabinosides ahead of rhamnosides.[65,67] One should therefore be aware that also the linkage position can have an influence on the retention.

The choice of the RP column is also important for the quality of the analysis. In studies where different RP columns have been compared, large variations are found in the quality of separation between free and conjugated flavonoids.[68] A C_{18}-RP column that is well end-capped is to be preferred, because residual silanol groups cause deterioration of the separation of flavonoids. Conventional reversed-phase LC columns (3–4.6 mm i.d.) are frequently used in combination with a flow splitter to make the flow rates compatible with the mass spectrometer. A major part of the mobile phase can be passed through a UV diode-array detector instead of sending it to waste.[54,55,57,70] Flavonoids show high and characteristic UV absorption,[30] giving information on the type of flavonoid aglycone and its oxygenation pattern with a sensitivity comparable to that of MS detection. An on-column limit of detection (LOD; signal-to-noise ratio (S/N) = 3) of around 10 ng is attainable for LC/UV and LC/MS in the total ion current (TIC) mode, whereas an LOD <1 ng can easily be achieved in the single ion monitoring (SIM) mode using negative ionization.[51,55,57,61,69–71] Additional direct coupling of an NMR instrument to an LC/UV/MS system results in a unique and very powerful but expensive technique for the identification of complex unknown polyphenolic compounds of plant origin.[72,73]

MASS SPECTROMETRIC STRUCTURE CHARACTERIZATION

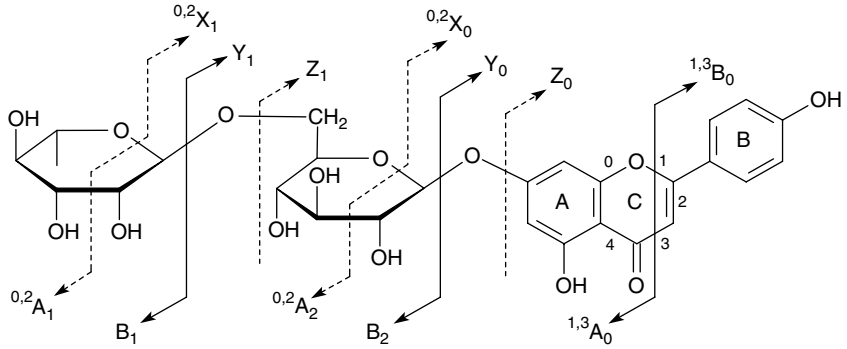
Flavonoid aglycone

Positive ion mode

Mass spectra obtained under electron ionization (EI) conditions have been widely used for structural investigations of flavonoids. This ionization technique has been applied to all classes of flavonoid aglycones.[74–76] However, complex fragmentations may occur during EI owing to the broad spread of internal energy carried by the initially produced $M^{+\bullet}$ ions, which may hide or suppress the $M^{+\bullet}$ ions and important primary fragment ions containing structural information. Furthermore, EI shows limitations for the analysis of polyphenolic flavonoids owing to their low volatility and high polarity. Following the introduction of fast atom bombardment (FAB) and atmospheric pressure ionization techniques (API), i.e. APCI and ESI, useful structural information on polar biomolecules could be obtained directly and easily. These techniques are especially powerful in combination with CID and MS/MS and, furthermore, have the additional advantage that the chemical hydrolysis and subsequent purification procedures that are necessary for the identification of the aglycone part can be circumvented.

The most useful fragmentations in terms of flavonoid aglycone identification are those that require cleavage of two C—C bonds of the C-ring, resulting in structurally informative $^{i,j}A^+$ and $^{i,j}B^+$ ions (Scheme 2). These ions can be rationalized by retro-Diels–Alder (RDA) reactions and are the most diagnostic fragments for flavonoid identification since they provide information on the number and type of substituents in the A- and B-rings. The flavonoid aglycone fragment ions can be designated according to the nomenclature proposed by Ma *et al*.[77] For free aglycones, the $^{i,j}A^+$ and $^{i,j}B^+$ labels refer to the fragments containing intact A- and B-rings, respectively, in which the superscripts *i* and *j* indicate the C-ring bonds that have been broken. For conjugated aglycones, an additional subscript 0 to the right of the letter is used to avoid confusion with the A_i^+ and B_i^+ ($i \geq 1$) labels that have been used to designate carbohydrate fragments containing a terminal (non-reducing) sugar unit (Scheme 2).[78]

The major routes of fragmentation resulting in A and B ions require cleavage of the C—C bonds at positions 1/3, 0/2, 0/3, 0/4 or 2/4 of the C-ring (Scheme 2). The fragmentation pathways depend strongly on the substitution pattern and the class of flavonoids studied, e.g. the additional hydroxyl group in position 3 of flavonols results in more and different possibilities for fragmentation compared with flavones. $^{0,2}A^+$, $^{0,2}A^+ - CO$, $^{1,4}A^+ + 2H$ and $^{1,3}B^+ - 2H$ are typically observed for flavonols, while $^{1,3}B^+$, $^{0,4}B^+$ and $^{0,4}B^+ - H_2O$ are found for flavones.[53,77] Figure 2 illustrates the low-energy CID spectra for the $[M + H]^+$ ions of (a) luteolin and (b) kaempferol. Although both flavonoids have the same molecular mass, the $^{i,j}A^+$ and $^{i,j}B^+$ ions allow the distinction between the flavone, luteolin, and the flavonol, kaempferol. The collision energy applied during CID processes plays an important role in the fragmentations observed. Ma *et al*.[77] reported a simultaneous decrease of the $^{0,2}A^+$ ion and increase of the $^{0,2}B^+$ ion for flavonols, while an increase in $^{1,3}A^+$ and decrease of $^{1,3}B^+$ was described for flavones by Wolfender *et al*.[53] on increasing



Scheme 2. Ion nomenclature used for flavonoid glycosides (illustrated on apigenin 7-*O*-rutinoside).[77,78]

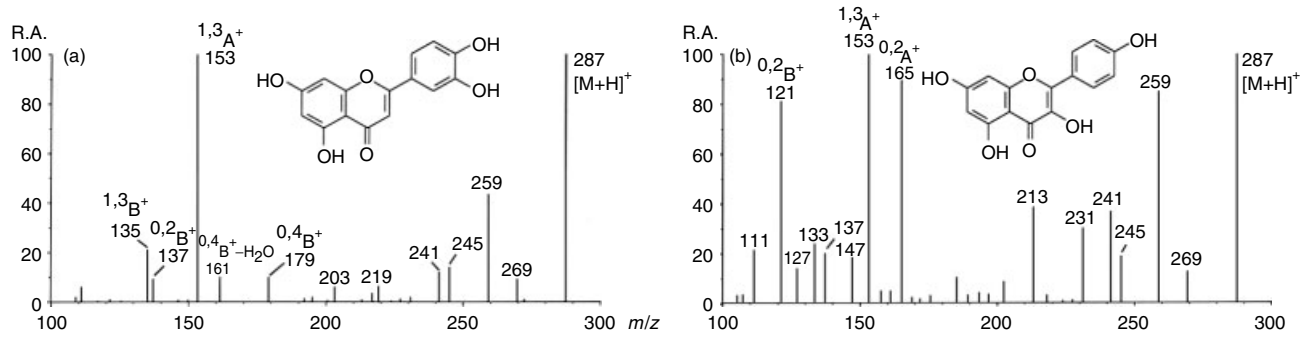


Figure 2. Low-energy CID spectra for the $[M + H]^+$ ions of (a) luteolin and (b) kaempferol obtained on a magnetic sector–quadrupole instrument equipped with a FAB source. (Reprinted with permission from Ref. 77. Copyright 1997 John Wiley & Sons, Ltd)

the collision energy. Both groups propose a collision energy of 30 eV to be most informative, giving the most all-round fragmentation. The $^{1,3}A^+$ ion, which is observed for all flavonoid groups, is generally the fragment most readily formed and often constitutes the most abundant fragment ion. Because the A-ring biogenetically originates from the acetate–malonate pathway (Scheme 1), giving rise to hydroxyl substituents at the 5- and 7-positions, the $^{1,3}A^+$ ion is most often found at m/z 153[53,77] or, in the absence of the 4-keto group, i.e. for flavanes and flavanols, at m/z 139.[53]

In addition to the $^{i,j}A^+$ and $^{i,j}B^+$ ions, discussed above, losses of small molecules and/or radicals from the $[M + H]^+$ ion are noted. Losses of 18 u (H_2O), 28 u (CO), 42 u (C_2H_2O) and/or the successive loss of these small groups are commonly observed. These losses are useful for identifying the presence of specific functional groups, i.e. a methoxy group is easily detected by the loss of 15 u (CH_3) from the $[M + H]^+$ precursor ion. The loss of a CH_3 radical appears to be prevalent so that the $[M + H - CH_3]^{+\bullet}$ ion dominates the whole spectrum.[53,77] This rather uncommon transition from an even-electron to an odd-electron ion is found to be characteristic of a phenolic methyl ether group. Losses of 56 u (C_4H_8) point to the presence of a prenyl substituent. Analysis of prenylated flavonoids using EI or FAB ionization has been reviewed by Takayama *et al*.[79] The 3-, 6- and 8-prenylated flavonoids can be differentiated by the difference in the fragmentation pattern obtained in EI, whereas the spectra recorded for *C*-6- and *C*-8-prenylated flavanones and chalcones are very similar owing to thermal isomerization between the flavanone and the corresponding chalcone in the ion source of the mass spectrometer prior to ionization. In FAB[80] and APCI,[81] thermal isomerization does not occur and all *C*-8-prenylated flavonoids can be differentiated from their *C*-6-prenylated positional isomers because the latter result in a more abundant loss of isobutene (56 u). $[M + H - C_4H_8]^+$ and $[M + H - C_4H_8 - C_4H_8]^+$ fragments are characteristic for diprenylated flavonoids, whereas $[M + H - C_9H_{16}]^+$ fragments are diagnostic of compounds containing a geranyl substituent.[81] Also, cyclic prenyl substitutions occur in flavonoids and have been analyzed by MS.[79,81]

Negative ion mode

In the structure analysis of flavonoids, positive ion CID spectra are most frequently used, whereas negative ion CID spectra are often considered to be more difficult to interpret. Higher collision energies are generally needed compared with the positive ion mode to yield adequate fragmentation and, sometimes, diagnostic ions observed in the positive ion mode and helpful in the structure determination are lacking. The negative ion mode is, however, more sensitive in flavonoid analysis,[54,55,57,59–63] and the fragmentation behavior is different, giving additional and complementary information. Cleavage of the C-ring by an RDA mechanism leads to $^{i,j}A^-$ and $^{i,j}B^-$ ions, providing information on the number and type of substituents in the A- and B-rings (Scheme 2). The MS analysis and fragmentation mechanisms of various aglycones in the negative ion mode have been studied in detail by Fabre *et al*.[63] The $^{1,3}A^-$ fragment is often the major fragment ion in the negative ion mode, whereas a $^{0,3}B^-$ ion is the major peak and characteristic for isoflavones.[82] The degree of hydroxylation on the B-ring has an impact on the fragmentation. For example, for flavonols containing two or more hydroxyl groups in the B-ring, e.g. quercetin, fisetin and myricetin, ions corresponding to $[^{1,2}A - H]^-$ and $[^{1,2}B + H]^-$ can be seen,[63,83] whereas in the case of an unsubstituted B-ring, the collision energy required to obtain fragmentation is much higher, leading to many product ions.[82] In some cases, a direct cleavage of the bond between the B- and C-rings, resulting in an $[M - B\text{-ring}]^-$ fragment, can be observed.[63] Losses of small neutral molecules, such as CO (−28 u), CO_2 (−34 u), C_2H_2O (−42 u) and the successive loss of these molecules, may also be prominent. Methylated compounds are characterized by the loss of 15 u resulting in an $[M - H - CH_3]^{-\bullet}$ radical ion which generally constitutes the base peak (Fig. 3).[63,83,84] Justesen[84] studied the fragmentation behavior of 10 deprotonated methoxylated flavonoids. Although isomeric compounds show a different fragmentation behavior, the exact position of the methoxy group could not be defined without comparison with standards or NMR analysis. The identification of prenylated flavonoids is hampered in the negative ion mode, because cleavage of the isoprenoid substituent cannot be observed.[81]

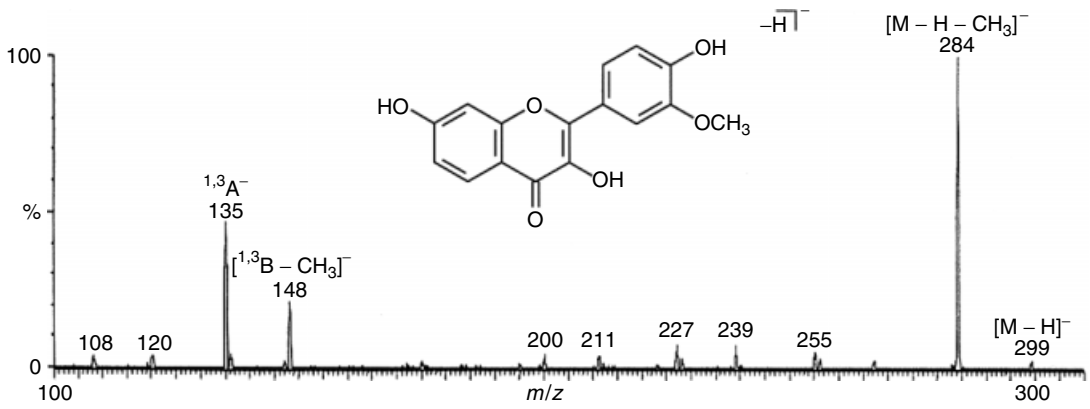


Figure 3. Low-energy CID spectrum of the $[M - H]^-$ ions of 3,4′,7-trihydroxy-3′-methoxyflavone obtained on a triple quadrupole instrument equipped with an ESI source. (Reprinted with permission from Ref. 84. Copyright 2001 John Wiley & Sons, Ltd).

FLAVONOID GLYCOSIDES

Differentiation between *O*-glycosides, *C*-glycosides and *O*,*C*-glycosides

Product ions from glycoconjugates are denoted according to the nomenclature introduced by Domon and Costello (Scheme 2).[78] Ions containing the aglycone are labeled $^{k,l}X_j$, Y_j and Z_j, where j is the number of the interglycosidic bond broken, counting from the aglycone, and the superscripts k and l indicate the cleavages within the carbohydrate rings. The glycosidic bond linking the glycan part to the aglycone is numbered 0. When the charge is retained on the carbohydrate residue, fragments are designated $^{k,l}A_i$, B_i and C_i, where i ($\geq$1) represents the number of the glycosidic bond cleaved, counting from the non-reducing end.

Differentiation between *O*-glycosides, *C*-glycosides and *O*,*C*-diglycosides can be made by examining first-order positive ion spectra or low-energy CID spectra.[85] The protonated *O*-diglycosides give rise to Y_1^+ and Y_0^+ ions (Fig. 4(a)), which are formed by rearrangement reactions at the interglycosidic bonds. Deuterium labeling experiments indicate that hydroxyl hydrogen atoms are required in the formation of Y_n^+ ions (Scheme 3).[85] In the case of *O*,*C*-diglycosides, only Y_1^+ ions, formed by fragmentation at the

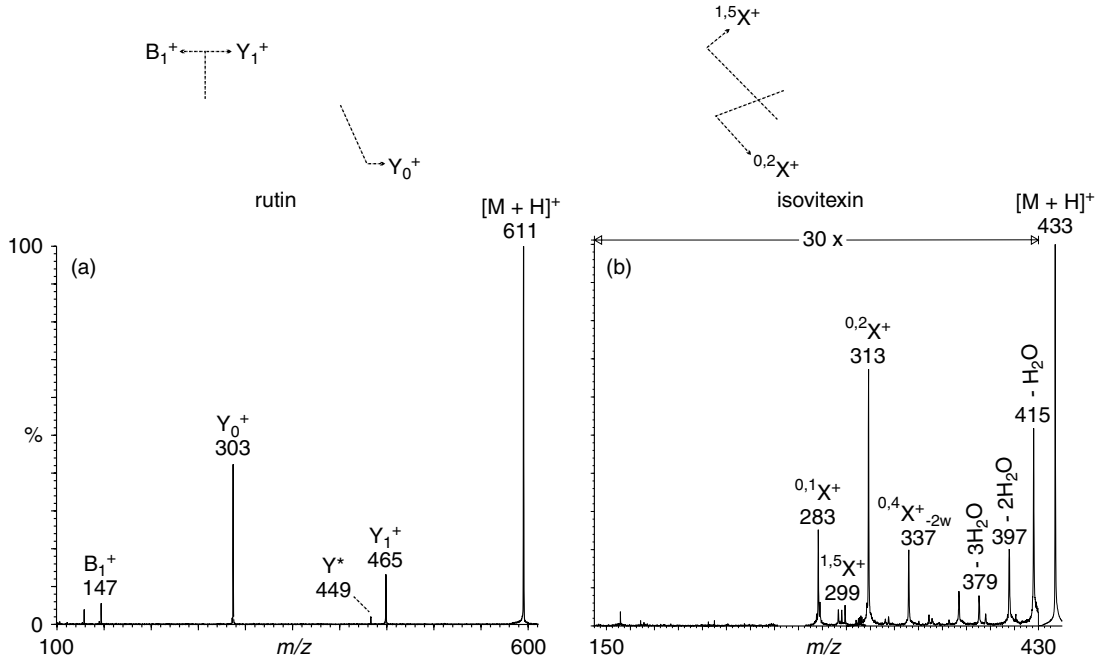
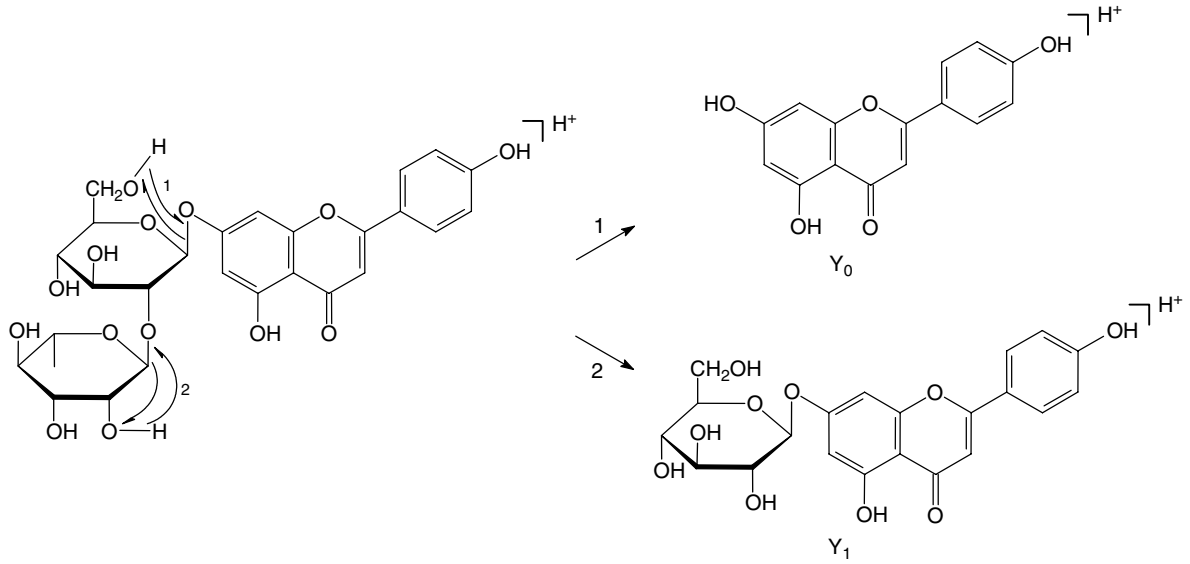


Figure 4. Low-energy CID spectrum of the $[M + H]^+$ ions of (a) rutin (quercetin-3-*O*-rutinoside) and (b) isovitexin (apigenin-6-*C*-glucoside) obtained on a magnetic sector-time-of-flight instrument equipped with an ESI source.



Scheme 3. Formation of Y_0 and Y_1 ions (illustrated on protonated apigenin 7-*O*-neohesperidoside). Any hydroxyl hydrogen atom from the sugar residues can participate in the rearrangement reactions shown. (Reprinted with permission from Ref. 85. Copyright 1994 John Wiley & Sons, Ltd).

JMS

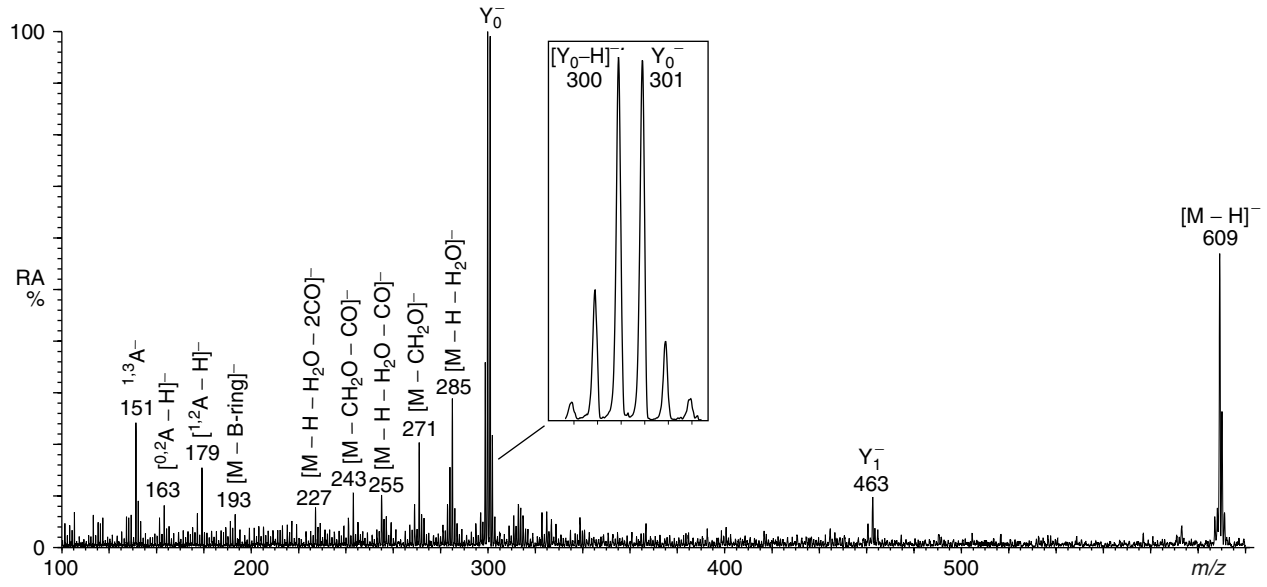


Figure 5. First-order LSIMS spectrum of rutin in the negative ion mode obtained on a magnetic sector-type instrument.

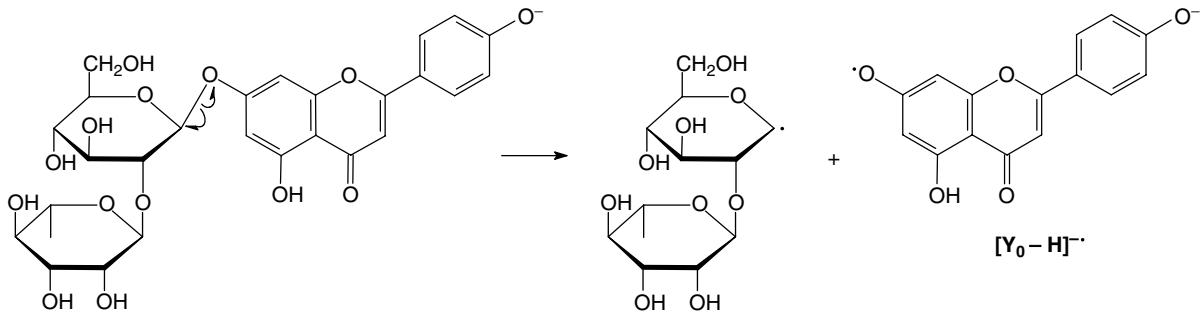
interglycosidic linkage, are detected, whereas, in the case of *C*-glycosides, only $[M + H]^+$ ions are observed together with cross-ring cleavages of the saccharidic residue and the loss of molecules of water (Fig. 4(b)).

Similar structural information can be inferred from mass-analyzed ion kinetic energy collision-induced dissociation (MIKE/CID) spectra obtained with negative ion FAB, as described by Becchi and Fraisse.[59] MIKE/CID spectra are obtained on older type instruments consisting of a magnet followed by an electrostatic sector, and are comparable to high-energy CID spectra obtained on more modern MS/MS instruments. In the spectra of *C*-glycosides, only the precursor $[M - H]^-$ ions are detected, whereas in the case of *O*,*C*-diglycosides, Y_1^- ions are also observed. A Y_1^- relative abundance >90% is characteristic of di-*O*,*C*-glycosides, while *O*,*C*-diglycosides show a very weak Y_1^- ion, but reveal a major product ion $[Y_1 - H_2O]^-$. Deprotonated *O*-diglycosides and di-*O*-glycosides give rise to both Y_1^- and Y_0^- ions.

Flavonoid O-glycosides

Mass spectrometric methods can be used to obtain information on the carbohydrate sequence and the aglycone moiety. In many cases, also the glycosylation position, the interglycosidic linkage position and the stereochemical identity of the terminal sugar residue can be defined. Cleavage at the glycosidic *O*-linkages with a concomitant H-rearrangement (Scheme 3) leads to the elimination of monosaccharide residues, i.e. the loss of 162 u (hexose), 146 u (deoxyhexose), 132 u (pentose) or 176 u (uronic acid), allowing the determination of the carbohydrate sequence.[86]

As an illustration, the low-energy CID spectrum of the $[M + H]^+$ ion (m/z 611) of rutin (quercetin-3-*O*-rutinoside), obtained on a magnetic sector-time-of-flight instrument equipped with an ESI source, is shown in Fig. 4(a). The loss of 146 u points to a terminal rhamnose unit and yields the Y_1^+ ion at m/z 465, while an additional loss of 162 u (hexose) or the direct loss of the rutinose residue results in the aglycone ion (Y_0^+) at m/z 303. In some cases, also the complementary monosaccharide ions can be detected, e.g. a terminal rhamnose unit results in an ion at m/z 147 (B_1^+). Also, aglycone fragments can sometimes be observed in the lower m/z range. To characterize the aglycone part of an unknown flavonoid, the $[M + H]^+$ high-energy CID spectrum and particularly the Y_0^+ high-energy CID spectrum are more suited, providing data similar to those discussed in Flavonoid aglycone section above. Exceptionally, also the first-order mass spectrum provides all information needed, as illustrated in Fig. 5 for quercetin 3-*O*-rutinoside (rutin), obtained using LSIMS in the negative ion mode. It is worth noting that in addition to the aglycone (Y_0^-) fragment, an abundant radical aglycone ($[Y_0 - H]^{-\bullet}$) product ion is also formed. The latter can also be observed in the high-energy $[M - H]^-$ CID spectra of quercetin *O*-glycosides.[87,88] The radical aglycone ($[Y_0 - H]^{-\bullet}$) product ion is formed by a homolytic cleavage as illustrated in Scheme 4, while the Y_0^- product ion is formed by a rearrangement analogous to that shown in Scheme 3. The radical cleavage of flavonoid *O*-glycosides resulting in odd-electron aglycone product ions has recently been examined in detail by Hvattum and Ekeberg[89] using negative ion ESI-MS/MS. The formation of a radical aglycone product ion is found to correlate with the antioxidant activity of flavonoids. Its abundance for flavones and flavonols depends strongly on the hydroxylation pattern of the B-ring and the nature and position of the sugar residues. The abundance ratio of the radical aglycone to the regular aglycone product ion originating from cleavage at the 3-*O*-glycosidic bond increases with increasing OH substitution on the B-ring, whereas the opposite holds for 7-*O*-glycosides. For flavanone and dihydrochalcone glycosides, no radical aglycone product ions are generated, indicating that a 2,3-double bond adjacent to the 4-carbonyl in the C-ring is required for the formation of stable radical aglycone product ions.



Scheme 4. Formation of the radical aglycone product ion ($[Y_0 - H]^{-\bullet}$) by a homolytic cleavage of the glycosidic bond between the aglycone and the glycan residue.

Sodium and potassium adduct ions are often detected in first-order mass spectra obtained with FAB or ESI in the positive ion mode. The alkali metals are generally extracted from glass during storage in solution and are more easily formed for flavonoids substituted at the 3-position, i.e., flavonol 3-*O*-glycosides and isoflavones.[62] Adduct formation can seriously hamper MS analysis, so that samples need to be desalted prior to analysis.[38] On the other hand, formation of alkali metal adducts for tandem mass spectrometric examination can provide valuable structure information, complementary to that obtained from the $[M + H]^+$ and/or $[M - H]^-$ CID spectra. Adducts with different alkali metals show different fragmentation patterns and the extent of fragmentation decreases in the order $Li^+ > Na^+ > K^+$.[90]

It is worth noting that the $[M + H]^+$ low-energy CID spectrum of rutin shows a weak product ion at m/z 449 (Fig. 4(a)). This rather irregular $[M + H - 162]^+$ ion (Y*) corresponds to the loss of the inner glucose residue. Y* is favored at lower collision energies[91] and can reach very high relative abundances,[92] which is very misleading since it yields false glycan sequence information. In this case, fragmentation of sodium-cationized or deprotonated molecules can provide the right glycan sequence information because these precursor ions do not have a mobile proton that can give rise to the loss of internal sugar residues.[93,94] On the other hand, Y* ions can be analytically useful since they can also provide information on the type of aglycone[92] or the differentiation between a neohesperidose or rutinose residue.[95] Neohesperidose (rhamnosyl-(α1 $\rightarrow$ 2)-glucose) and rutinose (rhamnosyl-(α1 $\rightarrow$ 6)-glucose) are the disaccharides most commonly found linked to flavonoids. Although they only differ by the interglycosidic linkage between the two monosaccharides, several MS methods allow the differentiation between both disaccharidic parts. On different magnetic sector type instruments, equipped with a FAB or ESI source, a larger Y_0^+/Y_1^+ ratio is obtained for isomers containing a neohesperidose than a rutinose substitution.[95] Using ESI ion trap MS/MS analysis, a much higher degree of fragmentation is found for deprotonated flavonoid *O*-neohesperidosides and the $^{0,2}X_0^-$ product ion has a m/z value at 146 u higher compared with *O*-rutinosides because the terminal rhamnose is not lost in the case of a 1 $\rightarrow$ 2 interglycosidic linkage.[92] Fragments at m/z 290 and 306, formed by elimination of water from B_2^+ product ions, are detected in the negative ion DCI quadrupole MS analysis of a flavonoid *O*-glycoside containing a 1 $\rightarrow$ 6 linked rhamnosyl-glucose and glucosyl-glucose part, respectively, whereas these ions are completely absent for their 1 $\rightarrow$ 2 linked analogues.[96]

An alternative method for the differentiation between flavonoid isomers consists in the addition of a metal salt and a neutral auxiliary ligand to flavonoids in solution, resulting in the formation of [M(II) (flavonoid-H) ligand]$^+$ complexes.[97] Complexes of flavonoids with divalent transition metals, especially Cu^{2+} and Co^{2+}, in combination with a neutral auxiliary ligand, 2,2′-bipyridine, have been used to achieve better LODs.[98] Additionally, they allow the distinction between flavonoid 7-*O*-rutinosides and 7-*O*-neohesperidosides: flavonoid 7-*O*-rutinoside–cobalt(II)–2,2′-bipyridine (1 : 1 : 1) complexes only lose the rhamnose and disaccharidic part during fragmentation, whereas the same complexes with flavonoid 7-*O*-neohesperidosides give an additional peak due to the loss of the aglycone and rhamnose, leaving the glucose attached to cobalt(II) and 2,2′-bipyridine. Also, the position of the dissaccharidic part can be identified in this way: for the 3-*O*-disaccharides of flavonoid–cobalt(II)–2,2′-bipyridine (1 : 1 : 1) complexes the loss of the disaccharide is dominant, while additional fragmentation pathways are followed for the complexes of 7-*O*-disaccharides.[97]

Flavonol 3,7-di-*O*-glycosides with glycan substituents of a different mass can easily be located because their protonated molecules more readily lose a glycan substituent located at the 3-position compared to the 7-position.[99] Similarly, a much more abundant aglycone product ion is detected for protonated luteolin 5-*O*-glucoside than for protonated luteolin 7-*O*-glucoside.[100] These findings are in full agreement with the susceptibility of the sugar–aglycone bond to acidic hydrolysis, which also depends on the attachment position of the sugar and shows the following order: 5 > 3 > 3′ = 5′ > 4′ > 7. The nature of the sugar has only a negligible influence on the strength of the sugar–aglycone bond.[101] High-energy CID can be applied to induce cross-ring cleavages of the sugar ring that sometimes is useful for obtaining information on linkage positions.[59]

Derivatization can be another approach to determine the location of a substituent, e.g. examination of the mass shifts of the A and B aglycone product ions after methylation provides information on the glycosylation

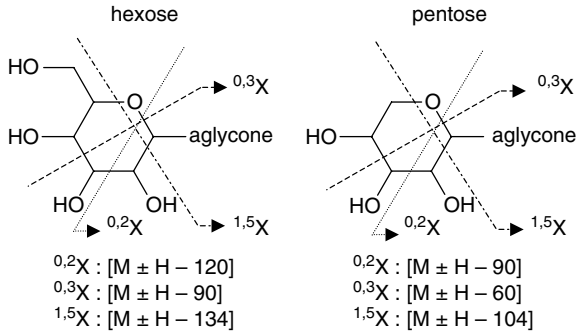
site.[102] A substituent on the A-ring is generally located at the 7-position since the 5-OH group shows a very low reactivity towards glycosylation because it is involved in intramolecular hydrogen bond formation with the 4-carbonyl group. Proof for the glycosylation site of the A-ring has been obtained by Chen *et al*.[103] by derivatizing the flavonoid with 2-chloroethanol, enzymatic hydrolysis and comparison of the LC retention time of the derivatized aglycone with that of a 5-hydroxyethylated and a 7-hydroxyethylated aglycone standard. An LC retention time corresponding to that of the 5-hydroxyethyl derivative indicates that the unknown was originally substituted at the 7-position.

The stereochemical structure of the terminal monosaccharide can be assigned without resorting to enzymatic or chemical hydrolysis prior to analysis.[104] For this aim, the analytes are acetylated, and stereoselective fragmentation of the peracetylated sugar-related fragments, generated by FAB or ESI in combination with cone voltage fragmentation, low- or high-energy CID, allow the identification of the monosaccharides most commonly encountered in flavonoid glycosides.

Flavonoid C-glycosides

In flavonoid *C*-glycosides, the sugar is directly linked to the flavonoid nucleus via an acid-resistant C—C bond. Therefore, the first-order mass spectra provide limited structural information except for the molecular mass. MS/MS analysis in combination with CID allows the characterization of *C*-glycosides in both the negative and positive ion modes. The major fragmentation pathways concern cross-ring cleavages of the saccharidic residue (Scheme 5) and the loss of molecules of water (Scheme 6), as illustrated in Fig. 4(b). To date, *C*-linked sugars have only been found at the C-6-and/or C-8-positions of the flavonoid nucleus (Fig. 1), so that the major challenge concerns the differentiation between 6-*C*- and 8-*C*-glycosyl flavonoids.

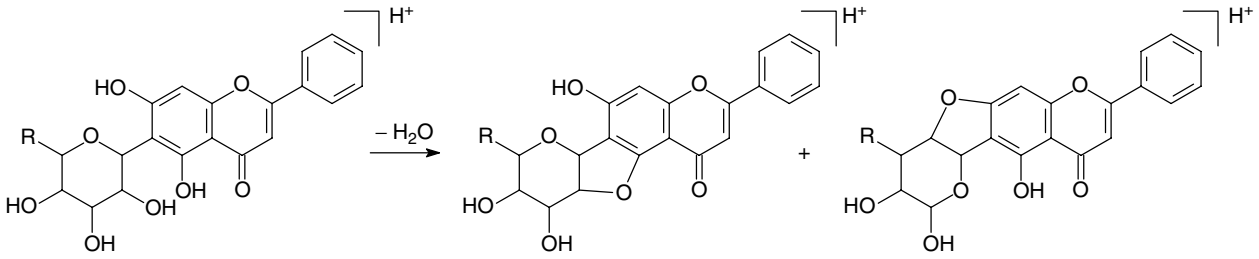
Both $[M+H]^+$ and $[M-H]^-$ ions lose molecules of water, being much more pronounced for 6-*C*- than 8-*C*-glycosyl flavonoids at high collision energies (Fig. 6).[59,105] 6-*C*-Isomers can give rise to elimination of water between the 2″-hydroxyl group of the sugar and the 5- or 7-hydroxyl groups of the aglycone, which is facilitated by hydrogen bonding between the ether oxygen atom of the sugar ring and the 5- or 7-hydroxyl group of the aglycone part, controlling



Scheme 5. Characteristic product ions formed by cross-ring cleavages in a hexose and pentose residue. The ± sign used here denotes + or −.

the rotation of the sugar residue. For 8-*C*-glycosyl flavonoids, a similar loss of water can occur only with the 7-hydroxyl group, a process that is counteracted by hydrogen bonding between the ether oxygen atom of the sugar and the 7-hydroxyl group of the aglycone (Scheme 6).[105]

The same fragmentation behavior is observed at low collision energies. The loss of a molecule of water is noted in the low-energy $[M-H]^-$ CID spectrum of isoorientin (luteolin 6-*C*-glucose), whereas it is absent from that of orientin (luteolin 8-*C*-glucose).[106] Similarly, the loss of two molecules of water is observed in the low-energy $[M+H]^+$ CID spectrum of acacetin 6-*C*-(6″-*O*-malonyl)glucoside, whereas it is absent from that of its 8-*C* analogue.[107] Waridel *et al*.,[108] on the other hand, found that the differences in water loss between 6-*C* and 8-*C*-glycosyl flavonoids in the positive ion mode are dependent on the collision energy and rather inadequate for structural assignment. Similar product ion spectra are obtained for the $[M+H]^+$ ions of 6-*C* and 8-*C*-isomers, except when the $^{0,2}X^+$ ($[M+H-120]^+$) fragment is selected as precursor ion. More extensive fragmentation is noted for the $^{0,2}X^+$ fragment of the 6-*C*- compared to the 8-*C*-glycosyl flavonoids.[108,109] Detailed guidelines for the identification and differentiation of 6-*C*- and 8-*C*-glycosyl flavonoids have been reported by Becchi and Fraisse[59] using high-energy CID of $[M-H]^-$ ions, Li *et al*.[105] using high-energy CID of $[M+H]^+$ ions and Waridel *et al*.[108] using low-energy CID on an ion trap and a quadrupole time-of-flight instrument. In di-*C*-glycosides, sugar residues of different mass can be located since the C_6-sugar residue shows more extensive fragmentation than the C_8-sugar residue.[59,110]



Scheme 6. Loss of water observed for 6-*C*-glycosyl flavonoids involving the hydroxyl group at the 2″-position of the sugar residue and the hydroxyl group at the 5-or 7-position of the aglycone. This process is facilitated by hydrogen bonding between the ether oxygen of the sugar residue and a hydroxyl group at the 5- or 7-position, respectively. (Reprinted with permission from Ref. 105. Copyright 1992 John Wiley & Sons, Ltd).

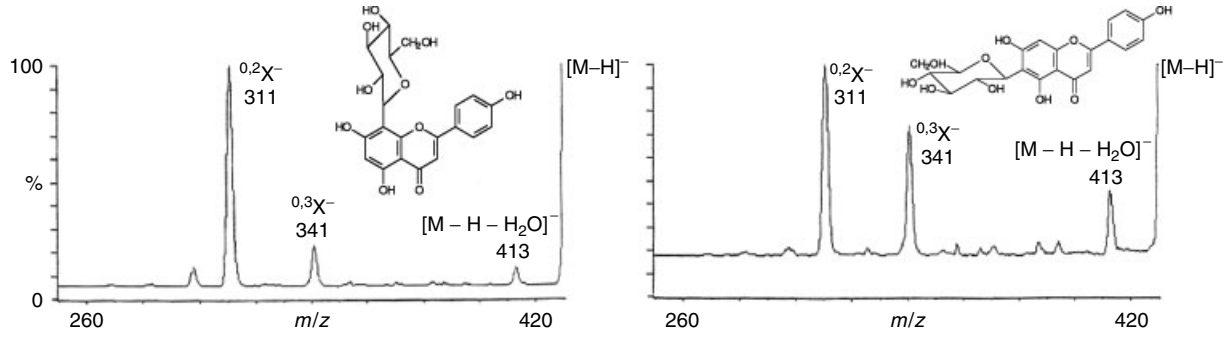
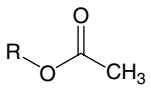
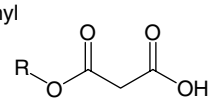
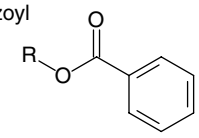
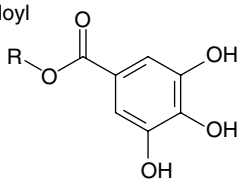
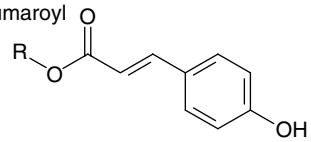
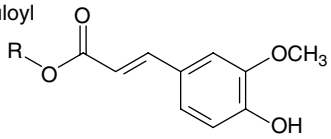
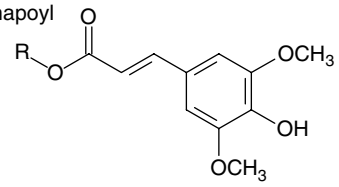


Figure 6. FAB MIKE spectra obtained for the $[M - H]^-$ ions of (a) vitexin (apigenin 8-*C*-glucoside) and (b) isovitexin (apigenin 6-*C*-glucoside). (Reprinted with permission from Ref. 59. Copyright 1989 John Wiley & Sons, Ltd).

Acylated glycosyl flavonoids

Several phytochemical studies have dealt with the analysis of plant extracts containing flavonoid glycosides with an acylated glycosyl part. Mass spectrometry is generally only used to obtain molecular mass information, but structure-specific information about the acyl group can be provided by neutral losses that are characteristic of the acyl group or the acylated glycosyl residue. Characteristic acyl-related product ions can be observed in the $[M + H]^+$ and $[M + Na]^+$ low-energy CID spectra and radical

Table 1. Acyl groups most commonly occurring in the glycosyl part of flavonoids and their characteristic product ions (the ± sign denotes + or −)

Acyl group	Characteristic fragments
Acetyl 	$[M + H - \text{acetylhexose}]^+$: −204 u[119,120]
Malonyl 	$[M \pm H - CO_2]$: −44 u[52,107] $[M + H - \text{malonyl}]^+$: −86 u[121] $[M + H - \text{malonylhexose}]^+$: −248 u[52,60,107,120–122]
Benzoyl 	$[M + H - \text{benzoylhexose}]^+$: −266 u[111] Benzoyl$^+$ at m/z 105[111] Benzoylhexose$^+$ at m/z 267[111]
Galloyl 	$[M \pm H - \text{galloyl}]$: −152 u[123–125] $[M - H - \text{gallic acid}]^-$: −170 u[124] $[M + H - \text{galloylhexose}]^+$: −314 u[123] $[\text{Gallic acid} - H]^-$ at m/z 169[124]
Coumaroyl 	$[M + H - \text{coumaroyl}]^+$: −146 u[126] $[M + H - \text{coumaroylhexose}]^+$: −308 u[126,127]
feruloyl 	$[M \pm H - \text{feruloyl}]^+$: −176 u[111,128] $[M \pm H - \text{feruloylhexose}]^+$: −338 u[111] Feruloyl$^+$ at m/z 177[111] Ferulic acid$^{+\bullet}$ at m/z 194[111] Feruloylhexose$^+$ at m/z 339[111]
Sinapoyl 	$[M \pm H - \text{sinapoyl}]^+$: −206 u[111,112] $[M \pm H - \text{sinapoylhexose}]^+$: −368 u[111,112] Sinapoyl$^+$ at m/z 207[111] Sinapic acid$^{+\bullet}$ at m/z 224[111] Sinapoylhexose$^+$ at m/z 369[111] Sinapoylhexose$^-$ at m/z 367[112]

acid-related product ions at high-energy CID conditions, which provide information on the presence and identity of the acyl group and its position on the flavonoid backbone structure.[111] In the latter study, acyl-related product ions could not be detected for deprotonated molecules, although a [sinapoylhexose − H]$^-$ fragment has been reported.[112] Table 1 gives an overview of the acyl groups most commonly encountered and their characteristic product ions. Other acyl groups, e.g. a caffeoyl[113,114] or cinnamoyl[113,115] group, can occur in the glycosyl part of flavonoids. Although similar fragmentations can be expected, detailed MS data are not available for these acylated flavonoids.

The exact location of the acyl group on the glycosidic part is difficult to define on the basis of MS data, but they appear to be predominantly located at the 6-position of a hexose moiety[116,117] although other positions can not be excluded. Only when a ^{0,4}X fragment is present in the spectrum can the location at the 6-position be confirmed.[107]

Caution should be exercized during storage and analysis of acylated glycosyl flavonoids because some of them are fairly labile, e.g. malonates are known to be prone to decarboxylation and especially de-esterification. Heating during extraction is generally unnecessary and can reduce the amount of acylated compound significantly.[52] Samples should be dried and stored preferably at −20 °C.[118]

CONCLUSIONS

Mass spectrometry has proven to be a very powerful technique in the analysis of flavonoids owing to its high sensitivity and the possibility of coupling with different chromatographic techniques, e.g. GC/MS, CE/MS and especially LC/MS, because it allows both qualitative and quantitative determinations. Structural information can be obtained on the flavonoid aglycone part, the types of carbohydrates or other substituents present, the stereochemical structure of terminal monosaccharide units, the sequence of the glycan part, interglycosidic linkages and attachment points of the substituents to the aglycone. Many of the structural characterization methods discussed are not restricted to flavonoid glycosides, but can also be applied to other glycosylated secondary plant metabolites (e.g. saponins, iridoid glycosides, chromon glycosides). Although some of the procedures described were established using older soft ionization techniques (e.g. FAB or LSIMS), they also hold for the newer techniques (e.g. ESI or APCI), since the fragmentation behavior of the molecular ion species generated with these techniques (e.g. [M + H]$^+$, [M + Na]$^+$ and [M − H]$^-$) are essentially the same.

In this review, both low- and high-energy CID methods were considered, and their specific merits discussed. Whereas methods based on low-energy CID permit the characterization of most structural features of flavonoid glycosides, methods based on high-energy CID provide additional structural information through more energetic fragmentation processes (e.g. cross-ring cleavages of saccharidic residues and homolytic glycosidic cleavages) and also more reproducible product ion spectra. With the recent developments of user-friendly LC/MS instrumentation that incorporates an ion trap or a quadrupole time-of-flight analyzer and allows low-energy CID, it can be envisaged that methods based on the latter technique will be more widely applied in the future.

The MS methods available at present rarely provide complete structure information, so that the search for novel MS approaches to the characterization of flavonoids and the implementation of new technologies to make the present methods faster, easier and more selective should continue. It is fair to state, however, that MS in combination with other spectroscopic techniques, e.g. UV and NMR, or directly coupled in an LC UV MS NMR system,[72,73] is a most powerful technique in the identification of complex unknown polyphenolic compounds of plant origin.

Acknowledgements

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