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Review

Application of mass spectrometry for identification and structural studies of flavonoid glycosides

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Abstract

Mass spectrometry is an important tool for the identification and structural determination of flavonoid glycosides. The advantages of mass spectrometry are high sensitivity and possibilities of hyphenation with liquid chromatographic methods for the analysis of mixtures of compounds. Different desorption ionization methods allow the analysis of underivatized glycosides. A review of mass spectrometric techniques applied to the identification and structural studies of flavonoid glycosides is presented.

Keywords: Flavonoid glycosides; Mass spectrometry; Liquid chromatography–mass spectrometry; Desorption ionization methods

Contents

1. Introduction

Flavonoid conjugates constitute a very diverse group of secondary metabolites in plants (Harborne, 1988a, 1988b). Most flavonoids in plant cells are present as glycosides. Sugar substitution on the flavonoid skeleton may occur through hydroxyl groups in the case of *O*-glycosides or directly to carbon atoms in ring A in *C*-glycosides. The number of sugar rings substituted on the aglycone varies from one to four. Another group of polyphenols present in the plant tissue,

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mostly in wood and bark, are the proanthocyanidins (condensed tannins), polymers of flavan-3-ols with different degree of polymerization (Haslam, 1981; Stafford, 1990).

These classes of secondary metabolites play an important role in interactions of plants with the environment (Berhow and Vaughn, 1999). Flavonoid glycosides and free aglycones are involved in interactions of plants with microorganisms, both pathogenic and symbiotic (Dixon et al., 1994; Spaink, 1995; Gianinazzi-Pearson, 1996). They also act as UV protectants in plant cells (Shirley, 1996), pigment sources for flower colouring compounds (Goto and Kondo, 1991; Weiss, 1991) and they play important roles in interactions with insects (Nahrstedt, 1989; Harborne, 1988a, 1988b). These plant metabolites also affect the human and animal health because of their significance in the diet, which is ascribed to their antioxidant properties (Rice-Evans et al., 1997), estrogenic action (Miksicek, 1993) and a wide spectrum of antimicrobial and pharmacological activities (Wollenweber, 1988; Weidenbörner et al., 1990; Dixon and Steele, 1999).

Due to the importance of flavonoids and their glycosides to and in living organisms, the identification and/or structural determination of such compounds occurring in plant tissue or other biological systems play an important role in many areas of science, particularly in plant science. Mass spectrometry (MS) is one of the physico-chemical methods applied to the structural determination of organic compounds. The high sensitivity and possibilities of hyphenation with chromatographic techniques sets MS among the most appropriate physico-chemical methods for the study of natural products from biological material. The characteristic features of MS is the use of different physical principles, both for sample ionization and separation of the ions, generated according to their mass (m) to charge (z) ratio (m/z). In this respect MS is different from other spectroscopic methods and it provides considerable flexibility in the detection, quantification, identification and structural determination of compounds.

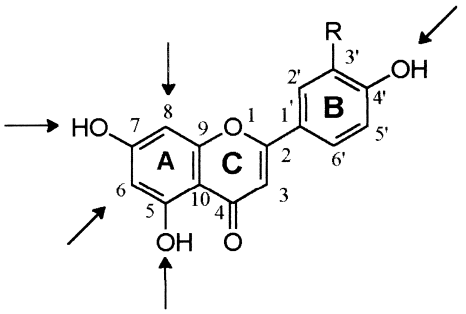
The application of MS to the analysis of flavonoid glycosides has increased with the new development of so called, "soft" ionization techniques. Compounds of this class are polar, non volatile and thermally labile. Electron impact (EI), with electron energies ranging from 10 to 100 eV, and chemical ionization (CI) were not suitable for MS analyses of underivatized flavonoid glycosides. Both methods require the analyte to be in the gas phase for ionization, and derivatization of the hydroxyl groups (methylation, trimethylsilylation and acetylation) was necessary. Only limited structural information about the sugar or the aglycone could be obtained for derivatized mono- and di-glycosides. With the introduction of desorption ionization techniques, the analysis of flavonoid glycosides without derivatization became possible. The first of these techniques, field desorption (FD) was developed by Beckey and Shulten (1975), but drawbacks related to the preparation of the sample for MS analysis, restricted its application. Another development was direct chemical ionization (DCI) (Hunt et al., 1977), in which the sample solution is deposited on the heated emitter placed directly in the reactant gas. The ions thus generated are desorbed and accelerated to the MS analyzer. In the early 80s, two new techniques were introduced: fast atom bombardment (FAB) (Barber et al., 1982) and liquid secondary ions mass spectrometry (LSIMS) (Aberth and Burlingame, 1984). An extension of these methods was the development of continuous flow fast atom bombardment (CF FAB/LSIMS) (Ito et al., 1985, 1986; Caprioli et al., 1986; Caprioli, 1990a, 1990b). In all three ionization methods, sample dissolved in a high boiling solvents is bombarded with high energy atoms (argon or xenon) or cesium ions, at energies of 8–30 keV. In parallel with these developments, other techniques were introduced, that were especially applicable to the combination of liquid chromatography with mass spectrometry. The most interesting, from the point of view of structural studies of flavonoid glycosides are thermospray (TSP) (Blakley and Vestal, 1983), and atmospheric pressure ionization (API) methods: electrospray (ESI) which is especially suited to the analysis of peptides and proteins (Whitehouse et al., 1985; Fenn et al., 1989), and atmospheric pressure chemical ionization (APCI) (Horning et al., 1974). The technical problems and the low sensitivity associated with the earlier introduced hyphenation techniques: the moving belt (McFadden, 1980) and particle beam (Bruins, 1985), limited seriously their applicability.

An excellent review by Arpino (1989, 1990) describes the LC/MS techniques introduced between 1980 and 1989. In addition, a number of papers that deal with the advances in instrumentation and applications of LC/MS systems for the analysis of mixtures of secondary metabolites from plants have been published (Maillard et al., 1993; Wolfender and Hostettmann, 1993, 1997; Sato et al., 1994; Wolfender et al., 1995a, 1997; Zhou and Hamburger, 1996; Vissers et al., 1997; Careri et al., 1998; Bruins, 1998; Niessen, 1998, 1999).

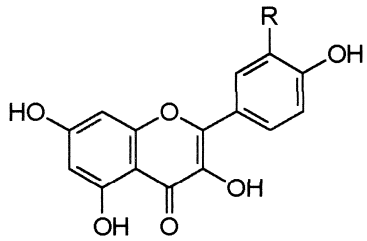
Flavonoid aglycones are structurally a rather diverse group of natural products. The most important variations in their structure arise from the level of oxygenation (hydroxyl or methoxyl groups) and the point of attachment of ring B (flavonoids and isoflavonoids (Fig. 1). The sugar component may consist of hexoses, deoxyhexoses or pentoses and in some cases glucuronic acids with the added possibility of *O*- or *C*-glycosidation. Differences in the configuration at the anomeric carbon(s) of the glycosidic units are also possible. The

consequence of this is that single MS technique will not provide all necessary structural information allowing an unambiguous assignment of the structure to an unknown compound.

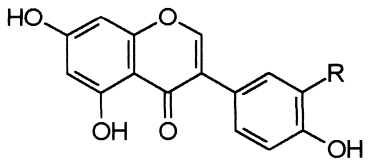
Fragmentation pathway of *O*-glycosylated flavonoids starts with the cleavage of the glycosidic bonds and elimination of the sugar moieties with charge retention on the aglycone or sugar fragments. In *C*-



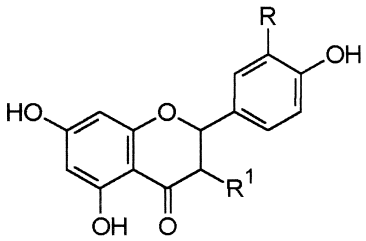
R = H or OH
flavone



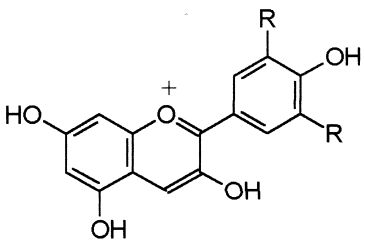
R = H or OH
flavonol



R = H or OH
isoflavone



R = H or OH, R1 = H flavanone
R = H or OH, R1 = OH didehydroflavonol



R = H or OH
anthocyanidin

Fig. 1. General structure of flavonoids and sites of their glycosylation and methylation, position of possible glycosilation of the flavone aglycone are indicated with arrows.

glycosides, mainly fragmentation of the sugar units is observed. In contrast to what is observed with EI techniques, in normal desorption ionization mass spectra fragmentation of the aglycone is not seen. With the later techniques (FAB, LSIMS, DCI) the information available is limited to the molecular mass of the compound, the size of the flavonoid molecule and the number of sugar units constituting the glycoside. In addition, with compounds containing two or more sugars, ions arising from the cleavage of the glycosidic bonds between sugar units are frequently weak.

The structural information can be enhanced by using tandem MS with collision induced decomposition (CID MS/MS) and desorption ionization techniques. Different experimental approaches for obtaining MS/MS spectra are known (McLafferty, 1983; Jennings, 1996). They are classified according to the translational energy of ions at which the collisions occur. The energy of ions leaving an ion source is dependent on the type of analyzer mounted in the mass spectrometer. In instruments equipped with an electromagnetic analyzer (magnetic-B and electrostatic-E sectors) high energy collisions (1–10 keV) take place. In two sector instruments, linked scans (E/B or B/E configuration) or mass analyzed ion kinetic energy (MIKE) experiments (only B/E configuration) are performed. High energy CID MS/MS analyses are run on three or four sector tandem mass spectrometers with both configurations of the analyzers mentioned above. On the other hand, in triplequadrupole and hybrid tandem instruments (electromagnetic and quadrupole analyzer), low energy collisions (energy below 100 eV) are involved. Tandem mass spectrometric experiments are also performed on instruments with ion trap analyzers (March, 1992), and in this case, it is possible to perform tandem experiments many times (MS^n) on sequential product ions. This is an interesting approach since it permits structural information from one single analysis.

With respect to flavonoid glycosides, the following information can be obtained from mass spectra measured using the techniques outlined above: (1) molecular mass, (2) structure of aglycone (pattern of hydroxylation on aglycone, point of attachment of ring B on ring C), (3) information about acylation of sugar hydroxyl groups and possible methylation or sulphation of aglycone hydroxyl(s), (4) number of sugar rings, their configuration and in some cases placement of glycosidic bonds. MS does not provide information about stereochemistry of the glycosidic linkage or distinguish between diastereomeric sugar units.

2. Electron impact and chemical ionization

Mass fragmentation pathways of flavonoid aglycones induced with electron bombardment ionization are well recognized (reviewed by Mabry and Ulubelen, 1980). EI mass spectrometry has been also applied to some extent for structural investigation of *O*- and *C*-glycosylated flavonoids. Prior to MS analyses, the compounds were methylated (Bouillant et al., 1975, 1978, 1979, 1980, 1984; Besson et al., 1979; Sakushima and Nishibe, 1988b), trimethylsilylated (TMS) (Wagner and Seligmann, 1973; Schels et al., 1977, 1978) or acetylated (Sakushima et al., 1980; Sakushima and Nishibe, 1988b). However, during methylation of flavanone glycosides, side reaction may take place and rearrangement of the flavonoid aglycone to chalcone sometime occurs (Shmid, 1972). In these cases, additional purification procedures for the isolation of the products are necessary.

Zinsmeister and coworkers studied the mass spectral behavior of TMS derivatives of mono-, di-, and tri-glycosides (Schels et al., 1977, 1978). Molecular ions were observed in the mass spectra, but the fragmentation pathway was dominated by fragment ions generated after expulsion of the TMS groups. Ions created after cleavage of the glycosidic bond with charge retention on the aglycone were also registered, while ions resulting from retro Diels-Adler reactions (RDA) were not observed. The site of the sugar substitution on the aglycone at C-7 and C-3 or C-5 could be determined from the relative intensities of the fragment ions created after the glycosidic bond cleavage. Ions characteristic for sugar fragments were also observed, but they did not bring any information about isomeric structures and form of the sugar ring. EI mass spectra registered from permethylated derivatives of flavonoid glycosides provided more structural information. Fragment ions from aglycones, including those coming from RDA reactions were observed, permitting elucidation of the structure of the flavonoid skeleton. Similarly, differentiation of hexoses, pentoses and glucoronic acid was also possible (Bouillant et al., 1975). The differences in the intensities of molecular and fragment ions in the mass spectra of some flavonoid glycosides were used to determine the location of the sugar ring (at C-7, C-3, or C-5). On the same basis, differentiation of sugar substitution at C-6 or C-8 of the flavonoid moiety was also possible in the case of *C*-glycosides (see Fig. 2). This was concluded from the differences in the relative intensities of $M^{+\cdot}$ and fragment created after cleavage of $CH_3O^{\cdot}$ radical $[M-31]^+$ in the spectra of permethylated (PM) isomeric *C*-glycosides of both luteolin and apigenin. Differences in the MS spectra of C-6 and C-8 disubstituted glycosides resulted from sequential elimination of sugar fragments, and in the case of two different sugars (hexose and deoxyhexose or hexose and pentose) it was possible to ascribe the positions at which they were substituted. On the contrary, only low intensities of

molecular ions were registered in the mass spectra of acetylated flavonoid glycosides. The fragmentation pathway was dominated by sequential elimination of ketene molecules or acetyl radicals (Sakushima et al., 1980).

The application of chemical ionization for studies of flavonoid glycosides did not give satisfactory results. When methane or ammonia were used as reagent gas, the protonated molecular ions were not observed in mass spectra of unmodified compounds. However, with the use of amines, it was possible to detect protonated molecules of several mono-*O*-glycosides (Mollowa et al., 1987). Although negative CI has also been used, only poor results were obtained, due to low

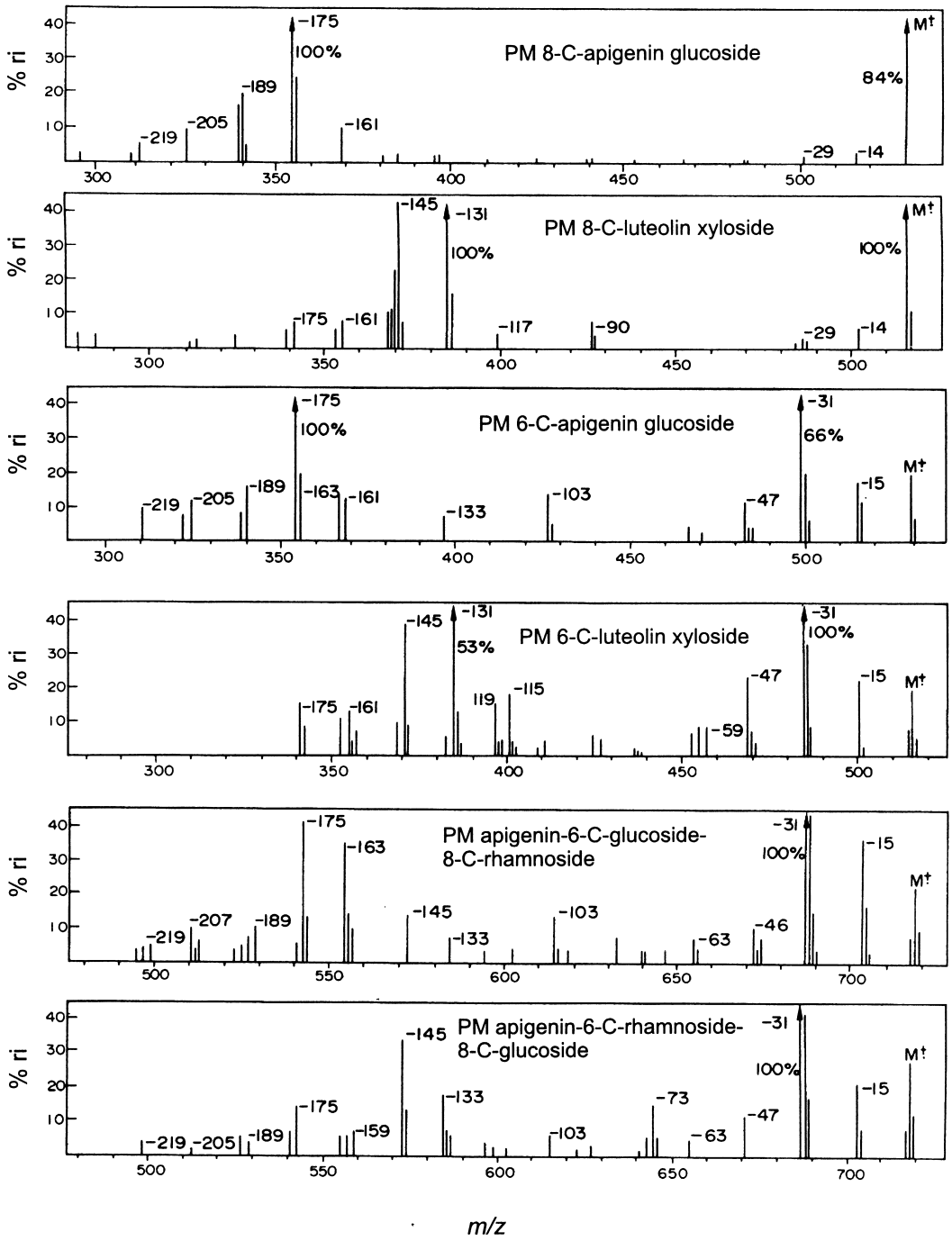


Fig. 2. Electron impact mass spectra of permethylated flavonoid *C*-glycosides. PM — permethylated compound (reprinted with permission from Bouillant et al., 1975).

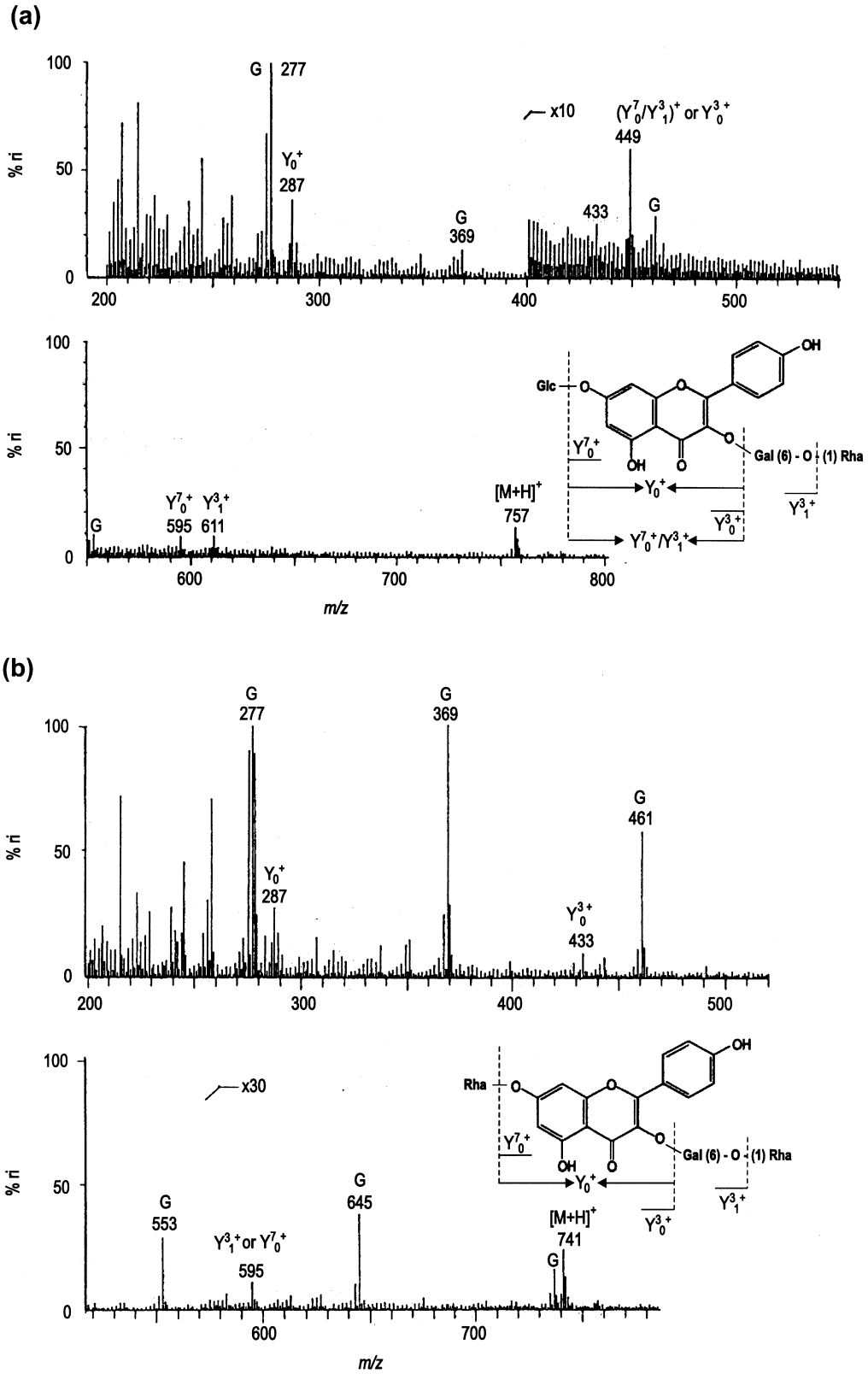


Fig. 3. Positive ions FAB mass spectra of flavonoid triglycosides: a — kaempferol 3-*O*-(6″-*O*-rhamnosyl)-galactoside 7-*O*-glucoside, b — kaempferol 3-*O*-(6″-*O*-rhamnosyl)-galactoside 7-*O*-rhamnoside (robinin). Y_n^{m+}: fragment ion created after cleavage of glycosidic bond between sugars; superscript describes the place of substitution on the aglycone; subscript describes the distance of the glycosidic bond from the aglycone; Y_0^+: an aglycon ion; $(Y_n^m/Y_n^m)^+$: fragment ion created after cleavages of two sugars substituted in two different positions on an aglycone. G: ions originated from protonated clusters of glycerol used as a matrix (reprinted with permission from Sakushima and Nishibe, 1988a).

intensities or lack of $[M + H]^+$ ions in the registered mass spectra (Itokawa et al., 1982).

3. Desorption ionization techniques

When applied to various unmodified flavonoid glycosides, desorption ionization techniques enabled the generation of mass spectra for molecules containing up to four sugar units, both in the positive and the negative ions mode. Intense ions are usually registered for protonated and/or cationized molecules $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$ (positive ions mode) or deprotonated molecules $[M - H]^-$ (negative ions mode). The fragmentation pathway follows the cleavage of glycosidic bonds in *O*-glycosides, consecutive expulsion of sugars is observed, and ions originating from the aglycones are also registered (Fig. 3). It should be noted however, that in the desorption spectra (FAB and LSIMS) of organic compounds usually ions of protonated clusters of matrix molecules are found, for example glycerol (G ions in Fig. 3). In the mass spectra of kaempferol triglycosides there were observed ions of the protonated cluster of glycerol molecules, used as matrix. As to *C*-glycosides, the fragmentation takes place in different ways. Mainly the cleavage of the sugar moiety is observed, sometimes with an additional fragmentation of the aglycone, according to the retro RDA. The relative intensities of the sample ions in the mass spectra registered with desorption ionization methods depend on the ionization technique used, the structure of the compound studied, and the purity of the sample.

3.1. Field desorption (FD)

This was the first technique applied for the analysis of intact flavonoid glycosides (Geiger and Schwinger, 1980; Domon and Hostettmann, 1985; Stein et al., 1985; Barbera et al., 1986). In all cases, the highest intensities were observed for ions of cationized molecules $[M + Na]^+$ and $[M + K]^+$, while ions of protonated molecules were of lower intensity. Fragment ions of different intensities, created after the consecutive loss of sugar moieties were also observed. It was possible to identify ions originating from the aglycone moiety, so the aglycone ions $[A]^+$, $[A + H]^+$ and $[A + Na]^+$ showed substantial intensities, in some spectra also $[A]^{2+}$ ions. During studies on acylated glycosides from *Bryum cappilare*, field desorption mass spectrometry was also used to confirm the substitution of the sugar moiety with a malonyl group (Stein et al., 1985).

3.2. Fast atom bombardment (FAB) and liquid secondary mass spectrometry (LSIMS)

There are numerous examples on the application of FAB and LSIMS for the analysis of flavonoid glycosides, both in positive and negative ion modes (Saito et al., 1983, 1985; Bridle et al., 1983; Domon et al., 1985; Crow et al., 1986; Sakushima et al., 1988; Stobiecki et al., 1988; Sakushima and Nishibe, 1988a; Becchi and Fraisse, 1989; Li et al., 1991, 1992a, 1992b; Wolfender et al., 1992; Li and Claeys, 1994; Claeys et al., 1996; Sumner et al., 1996). These ionization techniques were also used for structural studies of condensed tannins (Gujer et al., 1986; Self et al., 1986; Ohnishi-Kameyama et al., 1997). In the mass spectra, ions of protonated $[M + H]^+$ or deprotonated $[M - H]^-$ molecules of both classes of compounds were observed, as well as fragment ions. In the mass spectra of flavonoid glycosides, fragments were created after cleavage of bonds between sugars or sugar and aglycone, and in the later case between proanthocyanidin units.

The mass spectra recorded with both FAB and LSIMS techniques are often burdened with ions originating from the matrix used. Additionally, in some cases low signal-to-noise ratio (S/N) for protonated molecules and fragment ions is also observed. The matrices most often used (high-boiling solvents) are glycerol, nitrobenzyl alcohol (NBA), dithiothreitol/ditioerythritol, 5:1 w/w (magic bullet, DTT/DTE) and thioglycerol. The most suitable matrices are the first three mentioned solvents, because of long lasting signals, originating from the compounds analyzed, in the ion source of the mass spectrometer. This is especially important when CID MS/MS experiments are performed. A proper choice of the matrix affects also the yield of analyte ions sputtered from the solution (De Pauw, 1986; van der Peyl et al., 1985). Possible increase of the S/N ratio resulting for compound ions, improves significantly the quality of FAB spectra. Problems arising from the observed high intensities of matrix ions can be overcome with the use of continuous flow fast atom bombardment (CF FAB/LSIMS). This was clearly demonstrated by comparative analyses of rutin with LSIMS and CF LSIMS desorption techniques applied for registration of the mass spectra of flavonoid glycosides (Sumner et al., 1996), see Fig. 4.

Another strategy aiming to improve the S/N ratio in FAB spectra is methylation of the flavonoid glycosides prior to the MS analysis (Stobiecki et al., 1988). This approach provides also an additional information about the substitution pattern of sugars on the aglycone. The masses of partially methylated aglycone ions created after cleavage of the sugar moieties could easily be identified, the resulting difference in the mass between unmethylated and methylated aglycone

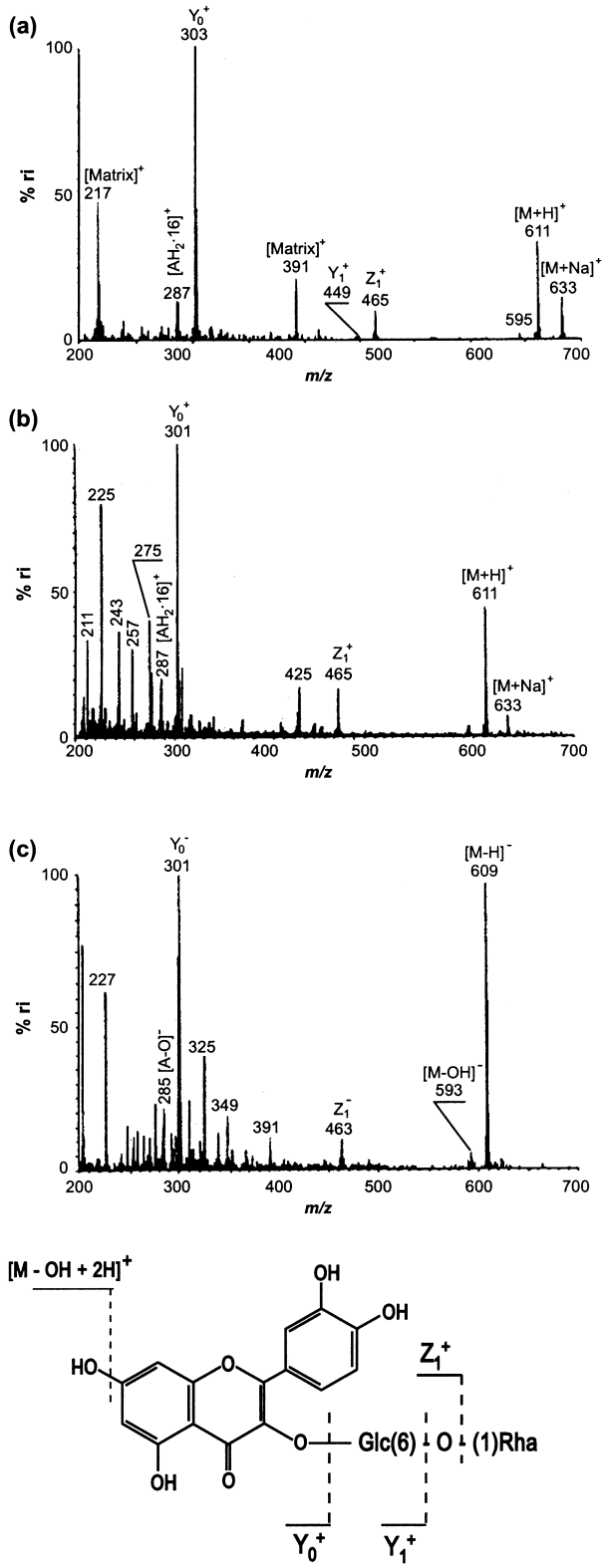


Fig. 4. CF-FAB mass spectra of rutin [quercetin 3-*O*-(6″-*O*-rhamnosyl)-galactoside]: a — positive ion LSIMS mass spectrum; b — positive ion CF LSIMS mass spectrum; c — negative ion CF LSIMS mass spectrum; Y_n^+ and Z_n^+ fragment ions created after cleavages of glycosideic bonds (reprinted with permission from Sumner et al., 1996).

enables the determination of the pattern of sugars substitution on the aglycone. The mass spectra of unmodified and permethylated quercetin 3-*O*-galactoside-7-*O*-glucoside are demonstrated on Fig. 5, the intense fragment ions (Y_n^+) were registered in the mass spectrum of permethylated glycoside.

The importance of the proper choice of matrix is also important because signals from protonated clusters of matrix molecules can overlap with ions of the protonated analyte molecules or with fragment ions originating from the flavonoid glycosides. In this case the use of a few different matrices is recommended. High concentration of sodium cations in the samples may also decrease the observed intensities of ions created from the molecules analyzed (Li et al., 1992b; Claeys et al., 1996). Thus, prior to MS analyses, samples of flavonoid glycosides should be purified on reversed phase C-18 cartridges with diluted acid as a solvent. The importance of such approach is illustrated in Fig. 6. Other impurities present in the samples, such as phthalates or polyethylene glycols, originating from the solvent for the isolation of the compound, also negatively influence the intensities of the analyte ions.

Hexoses, deoxyhexoses and pentoses could be differentiated in normal desorption (FAB or LSIMS) mass spectra of unmodified flavonoid *O*-glycosides, on the basis of m/z values of fragment ions created after cleavage of the glycosidic bonds. However, such fragment ions have often very low intensities and their identification between matrix ions is usually difficult. In such situations application of collision-induced dissociation tandem MS techniques (CID MS/MS) is very useful. For example, Crow et al. (1986) registered high energy CID MS/MS spectra of rutin and naringin on a three sector instrument (electric/magnetic/electric — E/B/E), where a collision cell was placed between the magnetic and the second electric sector. The comparison of normal and CID MS/MS spectra revealed substantial differences in the intensities of fragment ions created after the elimination of the sugar moieties. This difference was especially pronounced in the mass spectra of naringin, in which the glycosidic bond between rhamnose and glucose is situated at C-1 and C-2. Only $[M + H]^+$ ion at m/z 581 was observed in the normal FAB spectrum, while the fragments created after cleavage of the glycosidic bonds: Y_0^+ at m/z 273 and Y_1^+ at m/z 435, were registered during FAB CID MS/MS experiments. Similarly, structurally significant differences have been also found in the intensities of fragment ions created after degradation of the sugar ring in hesperidin (1–2 glycosidic bond) and neohesperidin (1–6 glycosidic bond) with the use of CID negative ion MS/MS technique (Fig. 7).

Other MS/MS techniques have also been applied for structural studies of flavonoid glycosides. Becchi and Fraisse (1989) demonstrated the usefulness of the col-

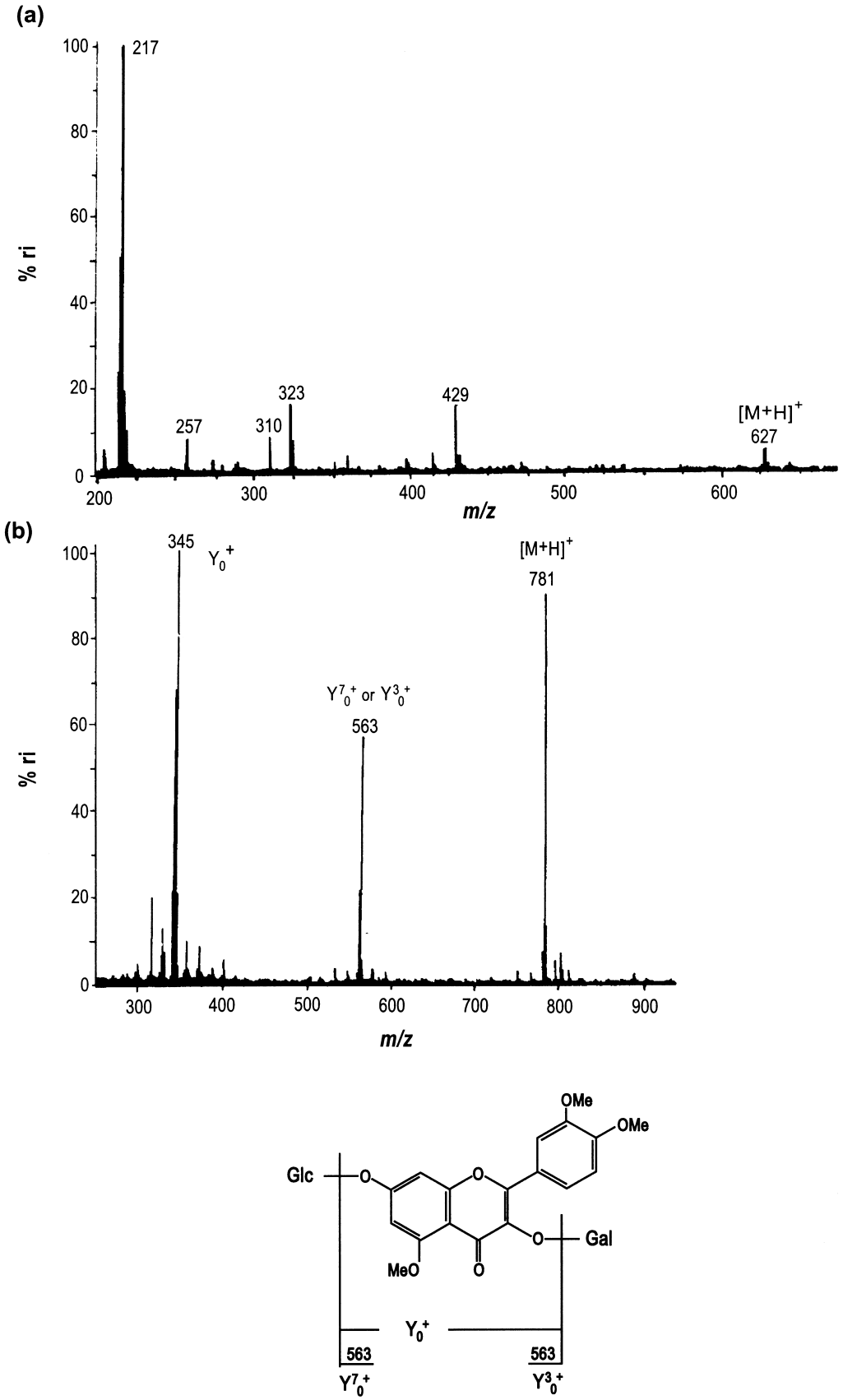


Fig. 5. FAB mass spectra of quercetin 3-*O*-galactoside 7-*O*-glucoside: a — unmodified; b — permethylated; Y_n^{m+} — fragment ions created after cleavages of glycosidic bonds (reprinted with permission from Stobiecki et al., 1988).

lision-induced dissociation mass analyzed ion kinetic energy (CID/MIKE) technique for the analysis of C-6 and/or C-8 mono- and di-glucosides isolated from plant tissues. In this case, the mass spectra in the negative ion mode were recorded on a reversed geometry (B/E) mass spectrometer. The same technique used Stobiecki et al. (1988) to analyse the permethylated quercetin 3-*O*-xylosyl-galactoside. On the other hand,

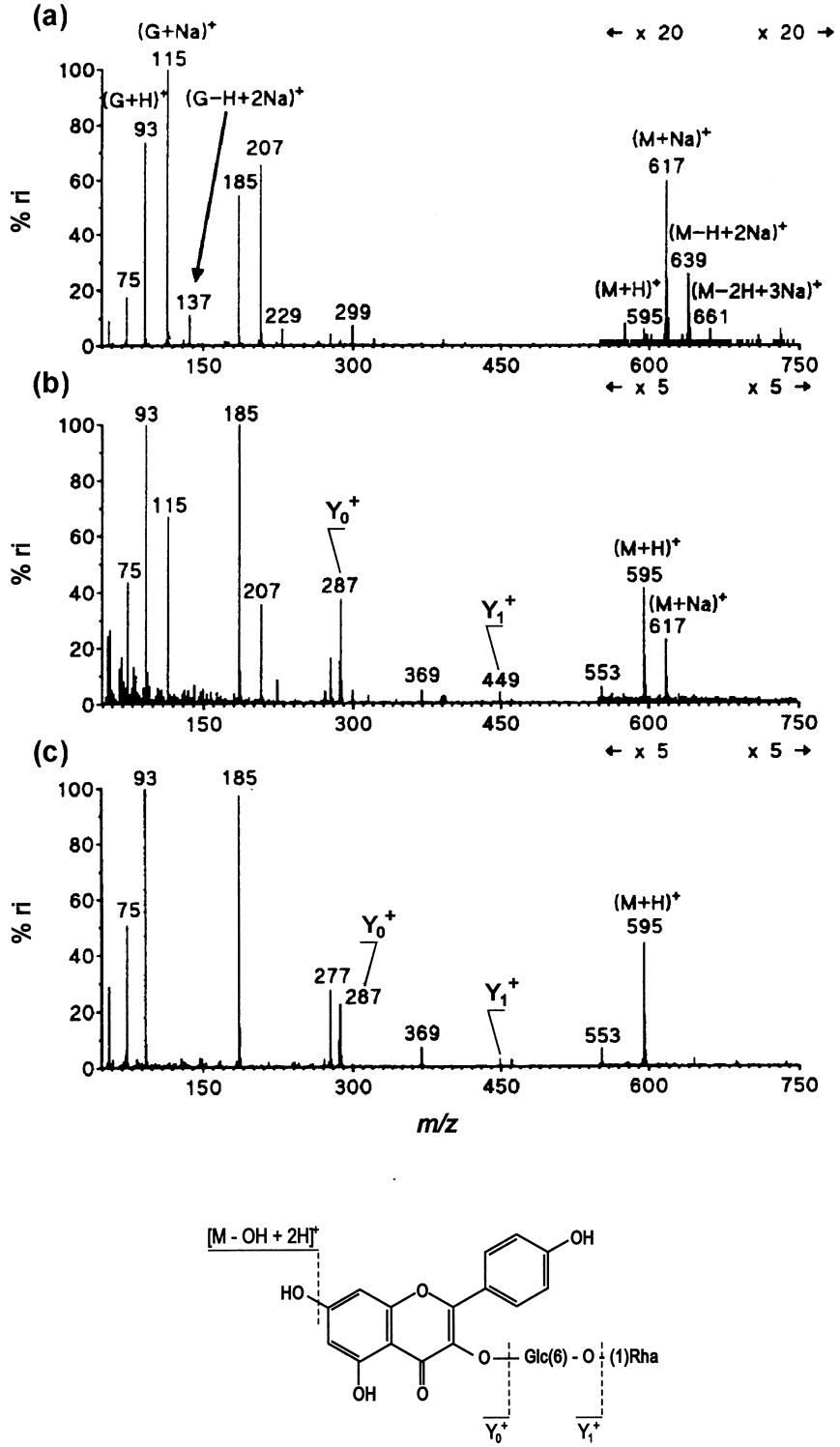


Fig. 6. FAB mass spectra of diglucoside kaempferol 3-*O*-(6″-*O*-rhamnosyl) glucoside: a — no desalting; b — desalted with deionized water; c — desalted with 0.02M HCl; Y_n^+: fragment ions created after cleavages of glycosidic bonds (reprinted with permission from Li et al., 1992b).

Claeys and co-workers (Li et al., 1991, 1992a, 1994; Claeys et al., 1996) applied a hybrid instrument with quadrupole as second analyzer (EBqQ) for characterization of *O*- and *C*-substituted flavonoid glycosides. In this case, the desorption ionization CID spectra were usually registered in a positive ion mode due to the better sensitivity observed. High and low energy experiments were performed. In the first case, collisions were induced with helium in the first field-free region with an energy of 8 keV and 50% beam attenuation, and product ions linked scan (B/E = const) mass spectra were registered. This enabled the determination of sugar substitution pattern of isomeric C-6 and C-8 glucosides, isoorientin and orientin respectively, on the basis of the abundance differences observed for the product ions in the CID MS/MS mass spectra (Li et al., 1992a) (Fig. 8). For recording low energy CID mass spectra precursor ions were selected at the resolution 1000 in a double focusing mass spectrometer; collisions were performed at an energy of 50 eV between the electromagnetic and the quadrupole analyzers. The low energy CID MS/MS spectra were recorded not only for protonated molecules of glycosides, but also for fragment ions, corresponding to aglycone ions created after cleavage of the sugar moieties. FAB CID MS/MS spectra with low energy collisions of protonated molecular ion of kaempferol rutinoside $[M + H]^+$ at m/z 595 and fragment ion of

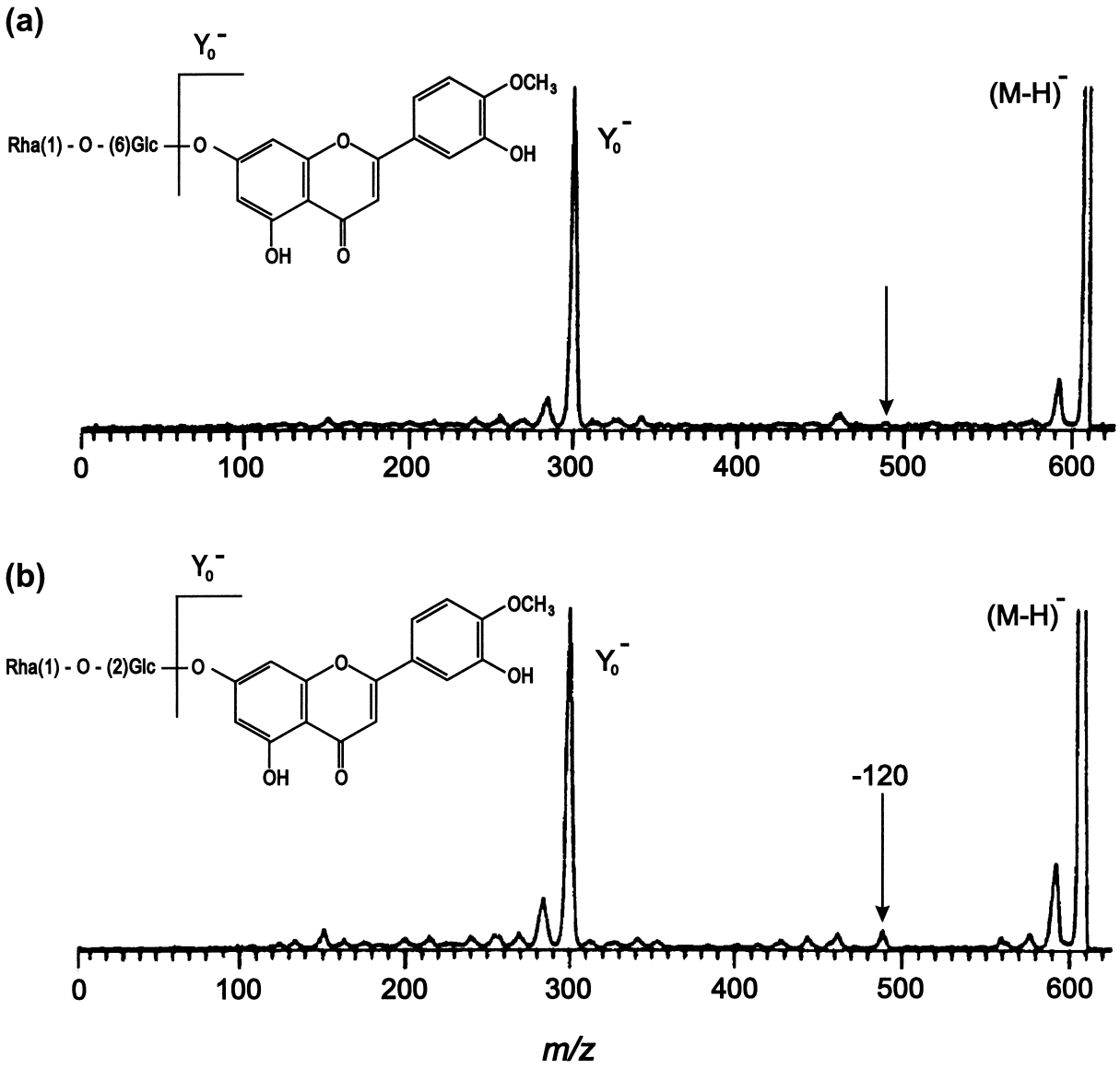


Fig. 7. CID MS/MS mass spectra: a — hesperedin — hesperetin 7-*O*-(6″-*O*-rhamnosyl) glucoside; b — neohesperidin — hesperetin 7-*O*-(2″-*O*-rhamnosyl) glucoside (reprinted with permission from Crow et al., 1986).

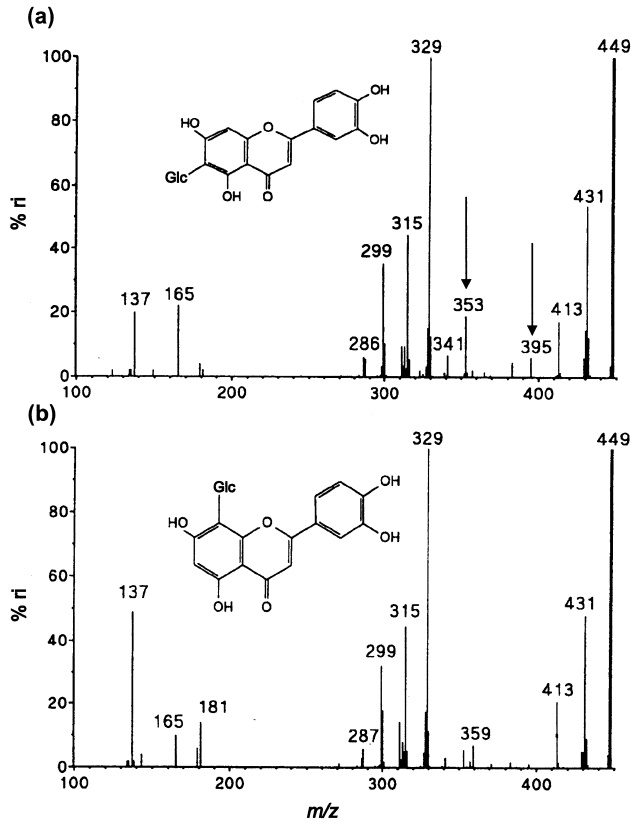


Fig. 8. High energy CID FAB mass spectra from $[M + H]^+$ ions at m/z 449 of *C*-glycosides: a — iso-orientin (luteolin 8-*C*-glucoside); b — orientin (luteolin 6-*C*-glucoside); (both isomers can be distinguished on the basis of abundances of the ions at m/z 395 and m/z 353 (reprinted by permission of Oxford University Press from Claeys et al., 1996).

apigenin at m/z 271 present in the mass spectrum of apigenin 7-*O*-neohesperidoside are demonstrated in Fig. 9.

Stobiecki and coworkers presented a methodological approach where different mass spectrometric techniques (LSIMS normal and CID linked scan spectra, EI GC/MS of chemically modified compounds) were applied for structural studies of flavonoid glycosides isolated from *Lupinus luteus* (Frański et al., 1999). The general scheme describing application of different mass spectrometric techniques in combination with the use of simple chemical modifications of compounds studied is shown in Fig. 10. In this approach, an unambiguous identification of aglycones and sugars, as well as the confirmation of the positions of glycosidic bonds, is achieved after GC/MS analyses of chemically modified compounds (permethylated, methanolyzed in 1 N HCl, and again methylated or acetylated). A similar approach for the determination of sugar substitution on flavonoid skeleton was proposed by Sakushima and Nishibe (1990). In this case, however, monotrimethylsilylated flavonoid methyl ethers, obtained from model compounds were analysed. Other strategies of chemical modification after hydrolysis were also applied. They involved reduction of sugars to alditols followed by acetylation, or trimethylsilylation of the obtained products. Such protocols were used for the elucidation of sugar moieties in anthocyanin and flavonol glycosides with GC/MS methods, respectively (Stobiecki et al., 1988; Glässgen et al., 1992c).

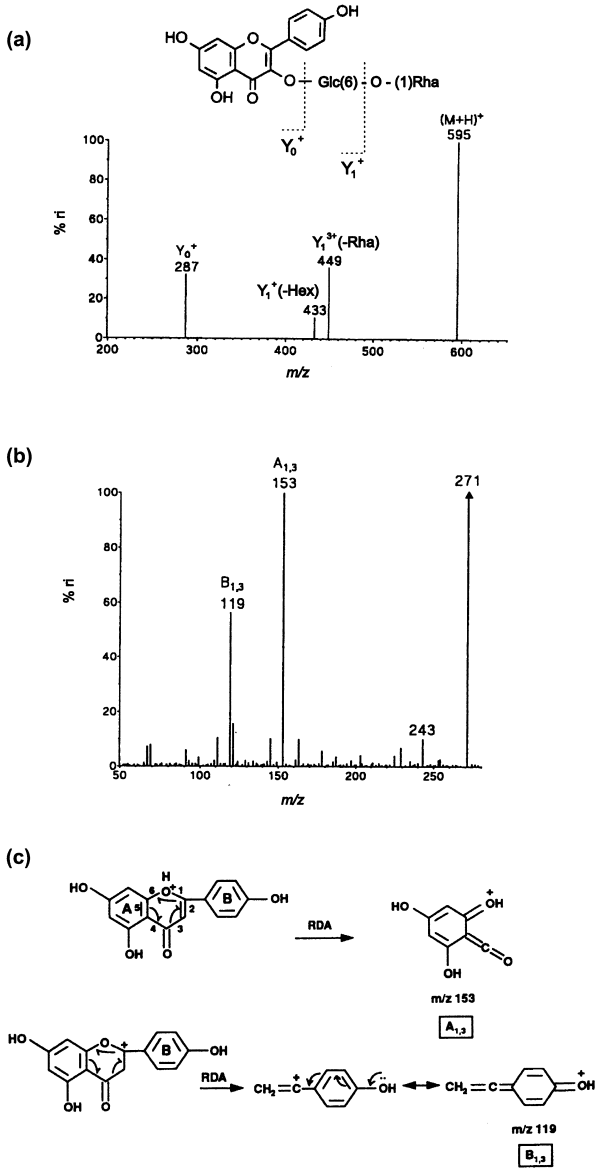


Fig. 9. Low energy FAB CID MS/MS mass spectra: a — from $[M + H]^+$ ions (595 m/z) of kaempferol 3-*O*-rutinoside; b — from Y_0^+ fragment ions (271 m/z) of apigenin neohesperoside; c — fragmentation pathway of apigenin; Y_n^+: fragment ions created after cleavages of glycosidic bonds (reprinted by permission of Oxford University Press from Clayes et al., 1996).

3.3. Direct chemical ionization

The DCI mass spectra of flavonoid glycosides in positive and negative ion mode were presented by Domon and Hostettmann (1985). The ionization method applied is applicable for flavonoid glycosides containing up to three sugar units, but the presence of glucuronic acid is excluded. When ammonia was used as reaction gas, protonated molecule ions $[M + H]^+$ and ammonium adduct ions $[M + NH_4]^+$ were observed in the mass spectra, and the presence of both types of ions, mentioned above was used to determine the molecular weight of the compounds studied. In this kind of mass spectra, fragments originating from the sugar moiety of the molecule have also been observed. Better results were usually obtained in the spectra registered in the negative ion mode (Domon and Hostettmann, 1985). Comparison of positive and negative ions DCI mass spectra of robinin showed some differences in the presence and/or relative intensities of the molecular and the fragment ions. The $[M + H]^+$ ion of the intact robinin molecule were not registered. Only in the negative ion mass spectrum was observed a peak of $[M - H]^{-\cdot}$ ions, and the main fragmentation pattern was related to the cleavage of the glycosidic bonds (Fig. 11). Flavonoid *O*- and *C*-glycosides were also analysed by the negative ion DCI spectra, using methane as a reagent gas (Sakushima et al., 1989). The signals from $M^{-\cdot}$ ions had fairly high intensities for monoglycosides, but decreased for di- and triglycosides. Fragment ions showing charge retention on sugar moieties were also identified with this method.

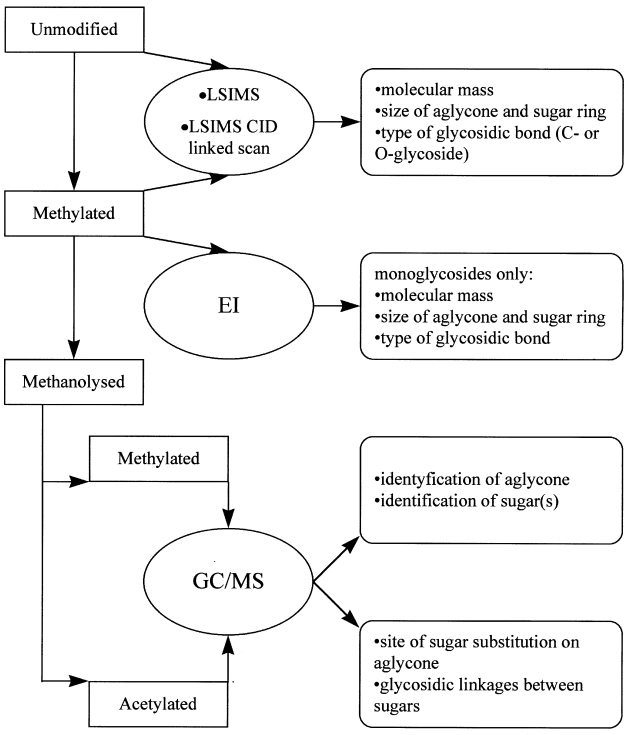


Fig. 10. Proposed strategy for the application of mass spectrometric techniques for structural studies of flavonoid glycosides.

Direct introduction electrospray mass spectrometry seems to be a valuable technique for the structural analysis of flavonoid glycosides. The mass spectra of pure compounds introduced in neutral solvents to the electrospray source are usually dominated by sodiated molecule ions $[M + Na]^+$, while protonated molecule ions $[M + H]^+$ often are not observed or have very low intensities. The intensity of cationized molecule ions can be decreased by acidification of the solvent used (Zhou and Hamburger, 1996; Stobiecki et al., 1999). The achievable sensitivity of ESI techniques may be even two orders of magnitude higher than those of FAB or LSIMS. Another advantage of electrospray ionization is better S/N ratio, due to lack of strong ions originating from the matrix in the spectral range above 200 amu. When using the ESI source, it is usually possible to register fragment ions created after consecutive cleavages of glycosidic bonds (Y_n^+ ions in Fig. 12). The intensities of fragment ions can be additionally increased by a collision induced dissociation in the source, achievable with an increase of the potential difference between nozzle and skimmer in the ESI source (Niessen, 1998, 1999).

In normal mass spectra of flavonoid glycosides registered with different desorption ionization techniques (LSIMS or FAB, DCI and FD) or direct introduction electrospray, the observed fragmentation pathways are very similar. Mainly glycosidic bonds are cleaved. However, in many cases the respective fragment ions are missing due to impurities present in the samples or to physico-chemical properties of compounds under study. In such a situation, CID MS/MS techniques are helpful. Additionally, the application of high and/or low energy collision experiments may give complementing results. The later technique is especially useful when the aglycone fragmentation has to be induced (Claeys, 1996), see Fig. 9.

4. Liquid chromatography–mass spectrometry

Technical developments in coupling mass spectrometers with liquid chromatographs, achieved during last decades, enabled the separation and identification of many classes of polar compounds, among others flavonoid glycosides, often present as complex mixtures in plant extracts. During the first attempts, only mixtures of free flavonoid aglycones, isolated from biological material, were efficiently analyzed on moving belt and particle beam interfaces with EI and/or CI ionization (Games and Martinez, 1989; Chavez et al., 1998). Application of these systems for analysis of fla-

vonoid glycosides did not give promising results, because of the polarity and thermolability of such compounds (Weinberg et al., 1992; Careri et al., 1998).

For a few years LC/MS systems have been applied for detection and identification of flavonoid glycosides in plants extracts or various biological fluids. Introduction of thermospray ionization interface (TSP) to LC/MS systems, has enabled the analysis of mixtures of polar flavonoid glycosides (Wolfender et al., 1995a, 1995b). However, the application of this technique has some limitations related to the thermal stability of the compounds studied. This is connected to the high temperature in the TSP ion source, which is necessary for efficient ionization of the molecules to be analysed. Additionally, optimization of the TSP ionization source parameters separately for each class of compounds in the mixture is necessary, as it strongly depends on the LC mobile phase composition and

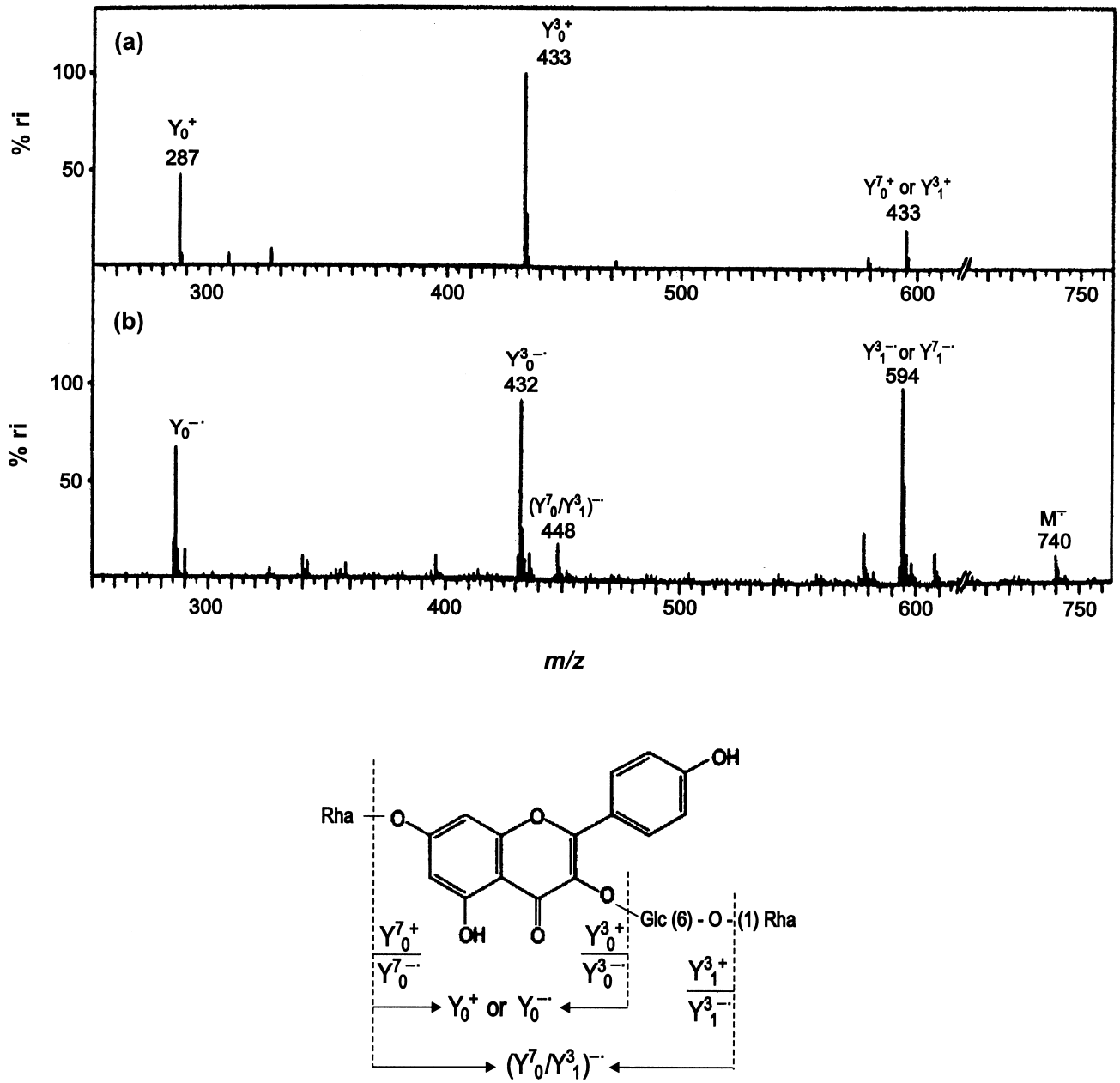


Fig. 11. DCI mass spectra of robinin [kaempferol 3-*O*-(6″-*O*-rhamnosyl)-galactoside 7-*O*-rhamnoside]: a — positive ions, b — negative ions (reprinted with permission from Domon and Hostettmann, 1985), $Y_n^{m+(-\cdot)}$: fragment ion created after cleavage of glycosidic bond between sugars; superscript describes the place of substitution on the aglycone; subscript describes the distance of the glycosidic bond from the aglycone, $Y_0^{+(-\cdot)}$ an aglycon ion, $(Y_n^m/Y_n^m)^{-\cdot}$: fragment ion created after cleavages of two sugars substituted in two different positions on an aglycone.

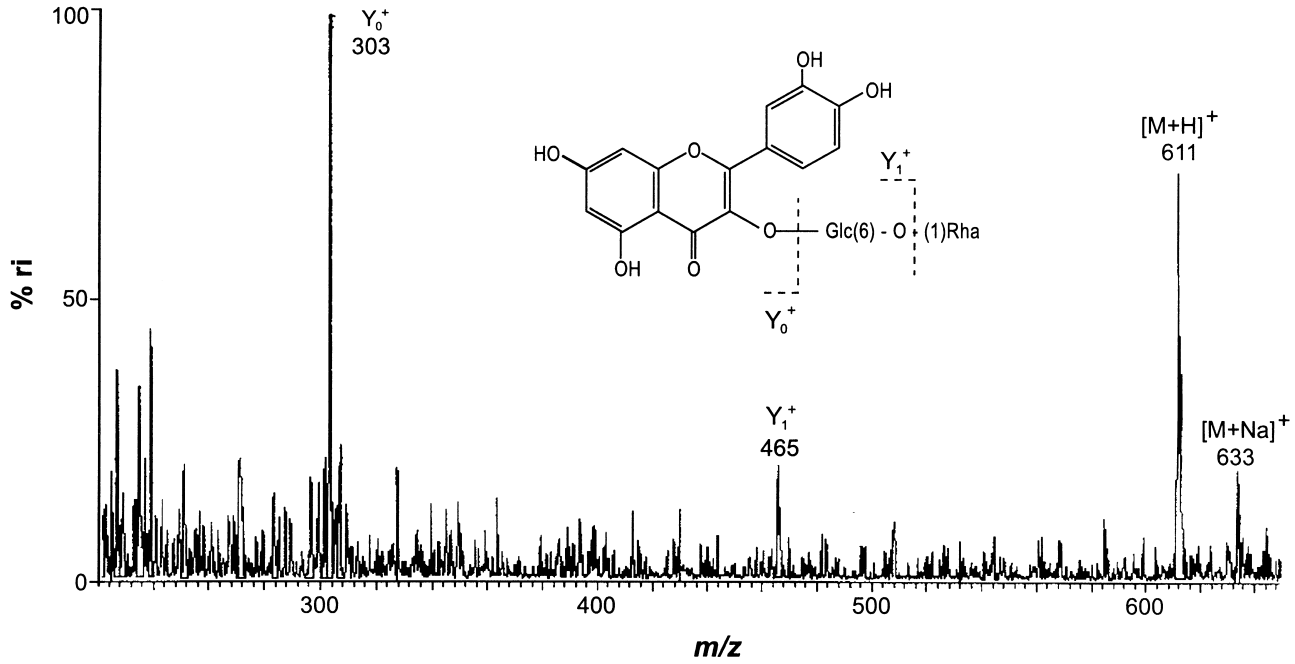


Fig. 12. Direct ESI mass spectrum of rutin [quercetin 3-*O*-(6″-*O*-rhamnosyl) glucoside]. Y_n^+: fragment ions created after cleavages of glycosidic bonds (reprinted with permission from Stobiecki et al., 1999).

flow rate. Attention has also to be paid to the following parameters: vaporizer and source block temperatures, ammonium acetate concentration in the mobile phase introduced to the ion source (possibly post-column addition to the mobile phase with an additional pump), and repeller potential. The mass spectra registered in the positive ion mode after TSP ionization are similar to those obtained with direct chemical ionization when ammonia is used as reagent gas. Such TSP spectra registered in positive or negative ion mode, provide information about the molecular masses of the

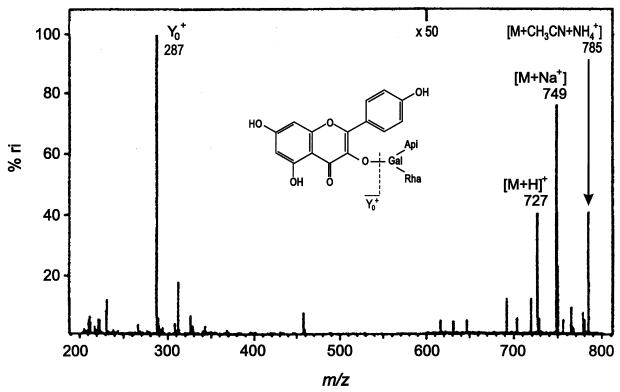


Fig. 13. TSP mass spectrum of triglycoside: kaempferol 3-*O*-[(2″-*O*-apiosyl) 6″-*O*-rhamnosyl]-galactoside. Y_0^+: fragment ions created after cleavages of glycosidic bonds (reprinted with permission from Wolfender et al., 1993).

flavonoid glycosides and the sizes of aglycone and sugars. Intensities of protonated molecule ions $[M + H]^+$ in the mass spectra are dependent on the number of sugars linked to the aglycone moiety. There is a substantial decrease in $[M + H]^+$ ions intensity with increasing number of attached sugar rings, a maximum of three sugar units may be present in the studied molecule. Cationized molecules with NH_4^+, Na^+, or K^+ cations, are often registered, and adducts with components of the mobile phase are also observed. Additionally TSP mass spectra usually give fragment ions giving sugar sequence information according to the size of the sugar rings (Fig. 13). LC TSP/MS has been used for the analysis of crude plant extracts from different sources. Hostettmann and coworkers applied the technique for the identification of secondary metabolites, among others flavonoid glycosides, in extracts from dried medicinal plants (Bashir et al., 1991; Maillard et al., 1993; Wolfender et al., 1993, 1995a, 1995b, 1997; Wolfender and Hostettmann, 1993, 1997). Pietta et al. (1994) carried out TSP/MS analyses of pure flavonoid glycosides obtained from *Ginko biloba* and *Calendula officinalis*, introduced directly or through liquid chromatograph to the ion source in the positive and the negative ion mode. LC TSP/MS was also successfully applied for detection and identification of a wide range of phenolic compounds, among others *O*- and *C*-flavonoid mono- and diglycosides in lemon peel (Baldi et al., 1995). Another example was LC TSP/MS analysis of the extract from

medicinal plant *Hypericum perforatum*, where a series of flavonoid glycosides was identified (Brolis et al., 1998). Other group used this LC/MS method for identification of monoglycosides in extracts from *Arnica montana* and *Arnica chamissonis* (Schröder and Merford, 1991). Green tea polyphenols and their glycosides were also studied with LC TSP MS/MS; a few flavonoid glycosides and catechins were identified (Lin et al., 1993; Poon, 1998).

Liquid chromatography with CF-FAB or CF-LSIMS interfaces has also been applied for the analysis of plant secondary metabolites. In this method only a very small volume of mobile phase eluted from the column enters the ion source of the mass spectrometer with maximum 10 μl/min delivered to the probe tip. During the analysis glycerol, used as matrix, was added to the mobile phase or delivered post-column to the eluate (Ito et al., 1985; Caprioli, 1990). This technique was used to analyse profiles of acylated isoflavonoid glycosides present in alfalfa (*Medicago sativa*) and chick pea (*Cicer arientium*). MS analyses were performed following purification of the compounds of interest on a polyamide column (Sumner et al., 1996).

Since electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were introduced in the field of combined liquid chromatography–mass spectrometry, the number of papers describing their usage has been increasing each year. Electrospray has become the most popular ionization method used. Atmospheric pressure ionization systems may be coupled to different mass spectrometric analyzers (quadroupole, ion trap, electromagnetic, time of flight and Fourier-transform ion cyclotron resonance), and thus, different designs of API interfaces for all kind of instruments are available (Niessen, 1998, 1999). Mobile phase flow in API interfaces may differ from nanoliters (so called nanoelectrospray) to 2 ml per minute. The temperature control of the APCI desolvation process is far less critical than in TSP-MS. In this way, a wide range of compounds may be analysed under the same conditions maintained at the APCI interface. For most mass spectrometers with electrospray ionization combined with LC, lower flow rates, up to maximum 100 μl/min are used. In many cases, splitting of the eluate from the LC column is necessary in order to decrease the volume of solution entering the API source. Such a flow rate enables an efficient desolvation process. Splitting of the column eluate provides also an opportunity to use double detection systems (UV and MS) of compounds present in the sample (Wolfender et al., 1995a, 1997, 1998). The mechanisms of electrospray and atmospheric pressure chemical ionization differ (Zhou and Hamburger, 1996). Both ionization processes are based on different physico-chemical effects. In ESI, ions are preformed in solution and this is essential for the next steps of ion formation in the gas phase. On the other hand, APCI relies on the gas phase chemistry where molecules have to be vaporized into the gas phase prior to ionization through charge or proton transfer. Additional feature of an electrospray ionization is in-source collision induced dissociation (CID) of protonated molecules obtained with potential difference (30–50 V) between nozzle and skimmer (Niessen, 1998). This enhancement of fragmentation may be also applied in structural elucidation of flavonoid glycosides. LC-ESI/MS has been applied for the identification of flavonoid glycosides isolated from different biological sources, for example in foods, (reviewed by Careri et al., 1998). Glässgen et al. (1992a, 1992b) used LC-ESI/MS and LC-ESI/MS/MS for the identification of anthocyanin glycosides in plant tissue and cell cultures of *Daucus carota*. More than 10 compounds were identified, among them mono-, di- and tri-glycosides. In some cases, terminal sugars were found to be acylated with sinapic, ferulic, or caffeic acid (Glässgen et al., 1992a, 1992b). The same LC-ESI/MS system was used for the detection and determination of the composition of flavonoids and their glycosides in *Oenothera* (Neumann and Schwemmle, 1993). The LC-ESI/MS system was also applied for a selective screening of 6′-*O*-malonylated or acetylated glucoconjugates present in plant tissues (fruits, roots, leaves) of species which are included in human diet (Withopf et al., 1997), or in foods prepared from soy (Barnes et al., 1994) and tomatoes (Mauri et al., 1999). Qualitative and quantitative analyses of free flavonoids and their glycosides in vegetables and beverages were performed with mixed, photo-diode array and mass spectrometric detection (Justesen et al., 1998; Ryan et al., 1999). The same analytical strategy was applied for the identification of flavonol aglycones and glycosides in berries (Häkkinen and Auriola, 1998). Finally, the first attempts for a parallel application of mass spectrometry, nuclear magnetic resonance and UV detection for the identification of flavonoid glycosides in crude plant extract have been reported by Hostettmann and co-workers (Hostettmann and Wolfender, 1997; Wolfender et al., 1998).

5. Conclusion and perspectives

During the last decade mass spectrometry has rapidly evolved to an instrumental method which can be used in chemical and biological laboratories. Many dedicated instruments are now available on the market, most often they are combined with liquid chromatography. Automation of both systems is readily achieved. The LC/MS systems can be applied for screening of flavonoid glycosides in plant tissue or biological fluids of different origin. The method is es-

pecially valuable when acylation of flavonoid glycosides is investigated.

Mass spectrometric techniques in combination with chemical derivatization of compounds may be applied for structural studies of flavonoid glycosides down to submilligram amounts.

New developments in the field of mass spectrometry facilitate the application of such methods in plant and medicinal sciences, but in some cases, the skill of an experienced mass spectrometrist, especially in CID MS/MS, is necessary.

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