Tandem mass spectral strategies for the structural characterization of flavonoid glycosides

F. Cuyckens[1], Y.L. Ma[1], G. Pocsfalvi[2] and M. Claeys[1*]

[1]*University of Antwerp (UIA), Department of Pharmaceutical Sciences, Universiteitsplein 1, B-2610 Antwerp, Belgium*
[2]*Centro Internazionale di Servizi di Spettrometria di Massa del CNR, via P. Castellino 111, I-80131 Naples, Italy*

In this article we review tandem mass spectral approaches to the structural characterization of flavonoid glycosides which are ubiquitous secondary plant metabolites. It is demonstrated how structural information on *O*-diglycosides can be obtained on (i) the carbohydrate sequence, (ii) the aglycone part, and (iii) the linkage in the diglycosidic part. Both the positive and negative ion modes have been employed.

Introduction

Flavonoid glycosides are predominant forms of naturally occurring flavonoids in plants, representing a large group of secondary plant metabolites [1-3]. They all contain a C_{15} flavonoid as an aglycone, are usually divided into *O*- and *C*-glycosyl flavonoids, are of interest because they have biological activities, are useful for chemotaxonomy, and are used as tracers in medicinal plant preparations. Recently, they have also received considerable interest in foodstuffs and nutraceuticals because of their antioxidant and anticancer properties [4].

During the last several years our research at the University of Antwerp has focused on the structural analysis of *O*-, *C*- and *O*,*C*-diglycosides isolated from medicinal plants using fast atom bombardment (FAB) or electrospray (ES) ionization in combination with collision-induced dissociation (CID) and tandem mass spectrometry (MS/MS) [5-10]. In this article we will review various tandem mass spectral approaches to the structural characterization of flavonoid glycosides. With regard to the structure elucidation of flavonoid *O*-diglycosides it will be illustrated how structural information can be obtained on (i) the carbohydrate sequence, (ii) the aglycone part, and (iii) the linkage in the diglycosidic part. Tandem mass spectral analyses have mainly been performed in the positive ion mode on protonated molecules generated by FAB or ES. In more recent work, however, which is briefly reported in the present article, we have also evaluated MS/MS methods on deprotonated molecules. This study was undertaken with a view to apply MS/MS in combination with ES-LC-MS for which the best sensitivity was obtained in the negative ion mode.

Experimental

The mass spectrometric data were obtained on different instruments: (1) a VG70SEQ instrument of EBqQ (E = electrostatic sector; B = magnetic sector; q = quadrupole gas cell; Q = quadrupole analyzer) configuration, (2) an Autospec-oa-TOF instrument of EBE-oa-TOF (TOF = time-of-flight analyzer) configuration, and (3) a VGZAB-T instrument of $B_1E_1B_2E_2$ geometry (all from Micromass, Manchester, UK). The acronym FAB was used to refer to caesium ion bombardment. The samples were dissolved in dimethylsulphoxide (DMSO) or methanol at concentrations of 1 mg mL^{-1} and 1 µL of the solution was mixed with 2 µL of the liquid matrix (glycerol) on the stainless steel probe tip. In the case of ES ionization, the flavonoids were dissolved in water/methanol (1:1; v/v) at a concentration of 100 µM and introduced into the ES source at a constant flow rate of 5 µL/min. Samples from plant origin were desalted using a solid phase RP-C18 cartridge in order to increase the $[M + H]^+$ signal intensity in the positive ion mode [11]. All the MS/MS spectra discussed here are CID product or metastable ion spectra. With regard to collisional-induced dissociation, both high- and low-energy CID has been evaluated on instruments (1) and (2). For details about the experimental conditions reference is made to our original articles [5-10]. Experimental conditions are only given below for experiments in the negative ion mode, which were performed on instrument (3). On this instrument high-energy CID spectra of FAB-generated deprotonated molecules were recorded at an accelerating potential of – 8 kV. The precursor ion was selected by the first two sectors and directed to the variable potential collision cell placed after E_1 to obtain product ion spectra. The collision cell was floated at a potential of – 4 kV. Argon was used as collision gas until 75 % attenuation of the precursor ion beam.

Article available at http://analusis.edpsciences.org or http://dx.doi.org/10.1051/analusis:2000280888

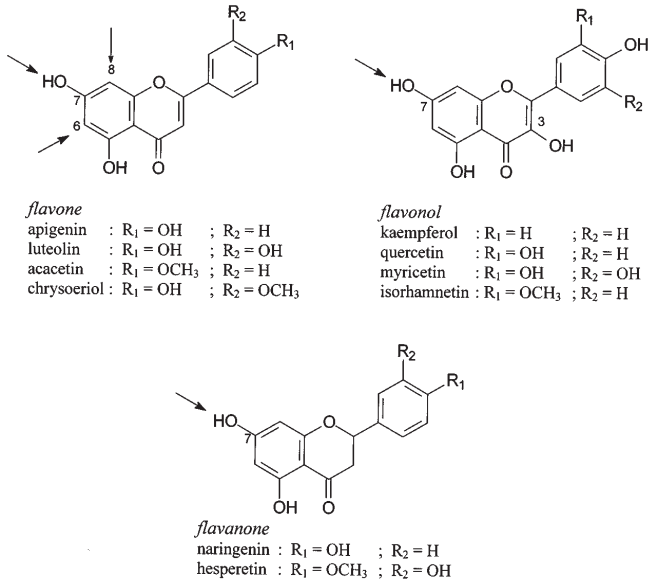


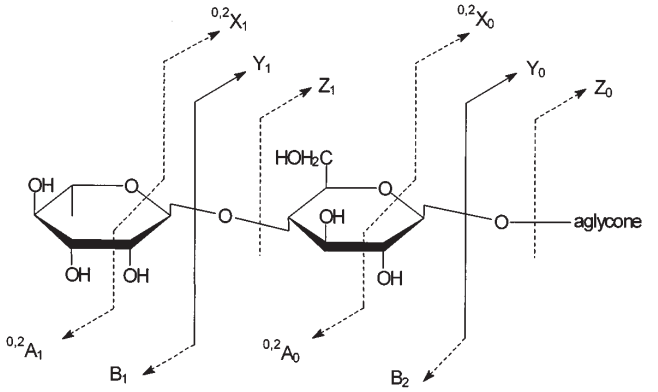
Figure 1. Structures of aglycones of the flavone, flavonol and flavanone type. Common glycosylation positions are indicated with an arrow.

Types of flavonoid glycosides studied

In this review we will focus on flavonoid glycosides having flavone, flavonol or flavanone aglycone moieties, since compounds containing these aglycones have most regularly been encountered. Structures of common flavones, flavonols and flavanones are given in figure 1. Flavonoid glycosides containing a flavonol or flavanone group generally occur as *O*-glycosides, whereas flavone glycosides can either correspond to *O*- or *C*-glycosides or be of a mixed *O*-*C*-diglycoside nature having a *C*-linked disaccharidic unit. The most common glycosylation positions are indicated in figure 1 with an arrow.

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

A differentiation between *O*-, *C*- and *O*-*C*-diglycosides can be made by examining first-order positive ion FAB spectra or low-energy CID spectra [7]. The protonated *O*-diglycosides give rise to Y_1^+ and Y_0^+ ions, which are formed by rearrangement reactions at the interglycosidic bonds. The carbohydrate ion nomenclature, which was introduced by Domon and Costello [12], is given in scheme 1. Deuterium labelling experiments indicated that hydroxyl hydrogen atoms are involved in the formation of Y_n^+ ions [7]. In the case of *O*-*C*-diglycosides only Y_1^+ ions, formed by fragmentation at the interglycosidic linkage, are detected, whereas in the case of *C*-glycosides only $[M + H]^+$ ions are observed.



Scheme 1. Carbohydrate ion nomenclature according to Domon and Costello [12].

Analogy exists for the MIKE/CID spectra obtained with negative ion FAB as described by Becchi and Fraisse [13]. In the spectra of the *C*-glycosides only the precursor $[M - H]^-$ ions are detected, whereas in the case of *O*-*C*-diglycosides also Y_1^- ions are 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 daughter ion $[Y_1 - H_2O]^-$. Deprotonated *O*-diglycosides and di-*O*-glycosides give rise to both Y_1^- and Y_0^- ions.

Characterization of flavonoid *O*-diglycosides in the positive ion mode

In this section selected data will be discussed to demonstrate how mass spectral methods can be used to obtain information on (1) the carbohydrate sequence; (2) the aglycone moiety and (3) the interglycosidic linkage type.

Figure 2 illustrates the metastable ion spectra (a) and low-energy CID spectra (b) of protonated rhoifolin and isorhoifolin. Rhoifolin and its isomer, isorhoifolin, only differ by the interglycosidic linkage type between the two monosaccharides rhamnose and glucose. However, this difference results in quite distinct spectra. The Y_0^+/Y_1^+ ratio is always larger for a 1→2 linkage than for a 1→6 linkage, where Y_1^+ corresponds to the loss of 146 units, indicating that the terminal monosaccharide is rhamnose, and Y_0^+ stands for the protonated aglycone.

Figure 3 shows the metastable ion spectra (a) and low-energy CID spectra (b) of protonated kaempferol-7-*O*-neohesperidoside and nicotiflorin. Again, the very different relative abundances of the Y_0^+ and Y_1^+ ions are worth noting.

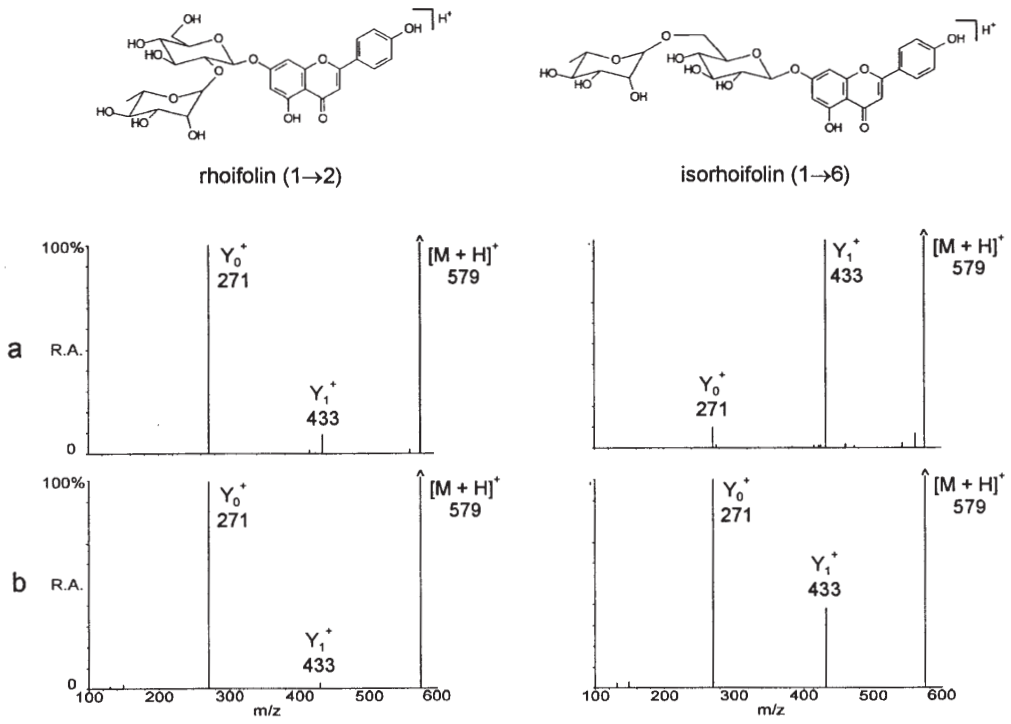


Figure 2. Metastable ion spectra (a) and low-energy CID spectra using helium as collision gas (b) of protonated rhoifolin (1→2) and isorhoifolin (1→6).

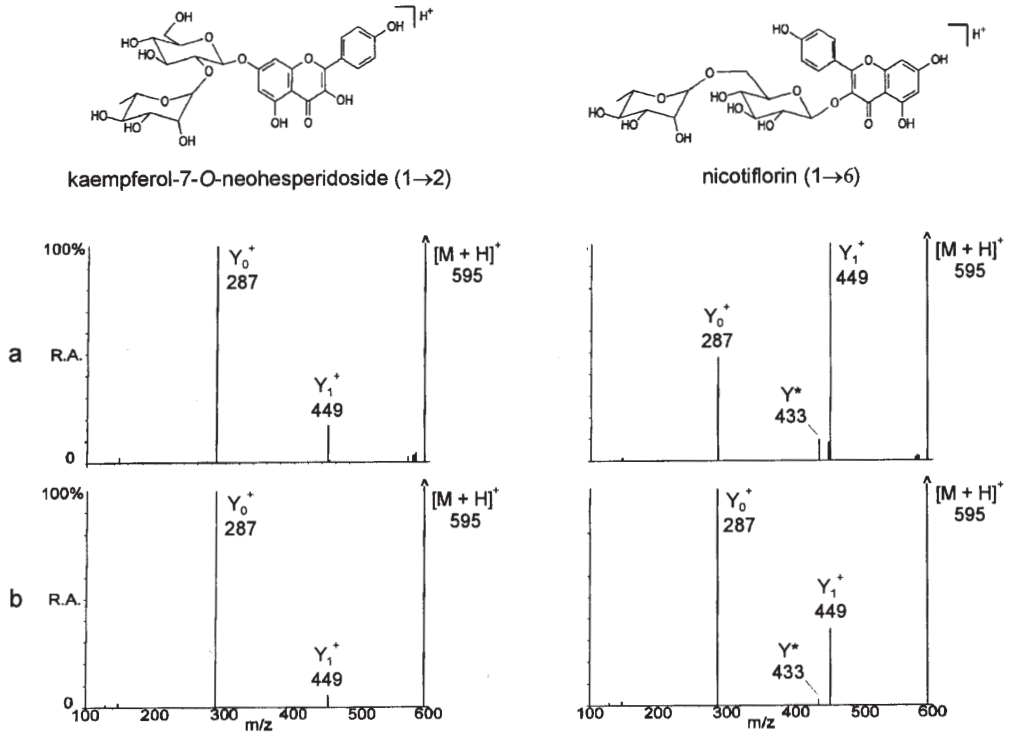


Figure 3. Metastable ion spectra (a) and low-energy CID spectra using helium as collision gas (b) of protonated kaempferol-7-*O*-neohesperidoside (1→2) and nicotiflorin (1→6).

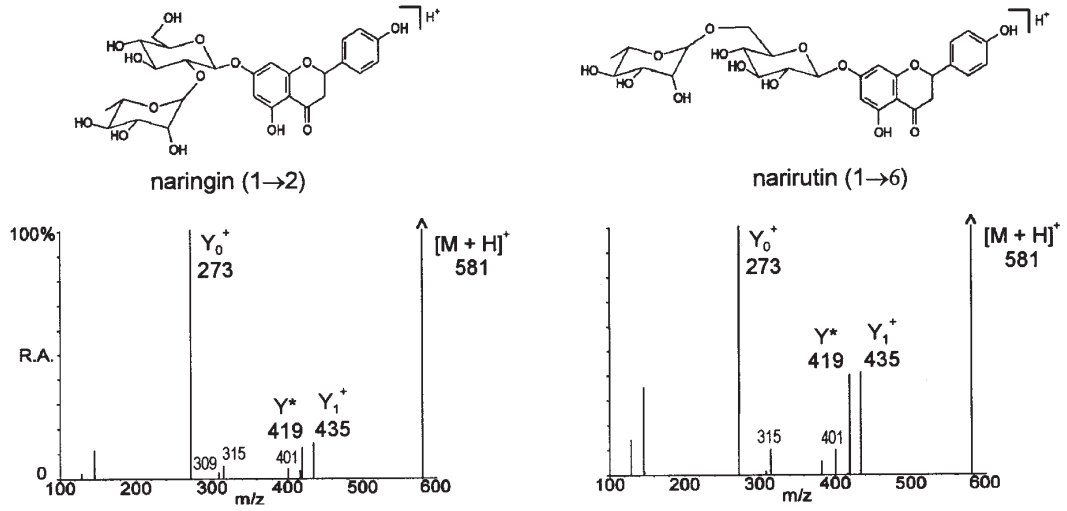


Figure 4. Low-energy CID spectra using helium as collision gas of protonated naringin (1→2) and narirutin (1→6).

Another significant difference is that a weak ion at *m/z* 433 (Y*) can only be seen in the spectra of nicotiflorin. This irregular Y* ion has been rationalized by loss of an internal dehydrated glucose residue, and a mechanism involving a mobile proton from the aglycone to the terminal rhamnose has been proposed [10]. The formation of Y* is twice as abundant using helium (E_{com} = 2,7 eV) as collision gas in comparison with methane (E_{com} = 9,8 eV), showing that its formation is favoured at relatively low collision energy.

Figure 4 illustrates the low-energy CID spectra of protonated naringin and narirutin. Naringin and narirutin both contain the flavanone, naringenin, and differ by the interglycosidic linkage type between rhamnose and glucose. It is worth noting that the irregular Y* ion, corresponding to the loss of an internal glucose residue, has about the same relative abundance as the Y_1^+ ion and is more abundant than in the case of nicotiflorin (Fig. 3). The more abundant Y* ion and smaller Y_0^+/Y_1^+ for naringin in comparison with narirutin is consistent with a 1→6 linkage.

Figure 5 shows the low-energy CID spectra of protonated rutin and hesperidin. Both compounds contain the same disaccharide part. Nevertheless, the Y^*/Y_1^+ ratio is much smaller for rutin, a flavonoid 3-*O*-rutinoside, than for hesperidin, a flavanoid 7-*O*-rutinoside. The additional peaks at *m/z* 431 (Y* – H_2O), 413 (Y* – $2H_2O$) and 345 ($^{0,2}X_0^+$) are also characteristic of flavanone *O*-diglycosides.

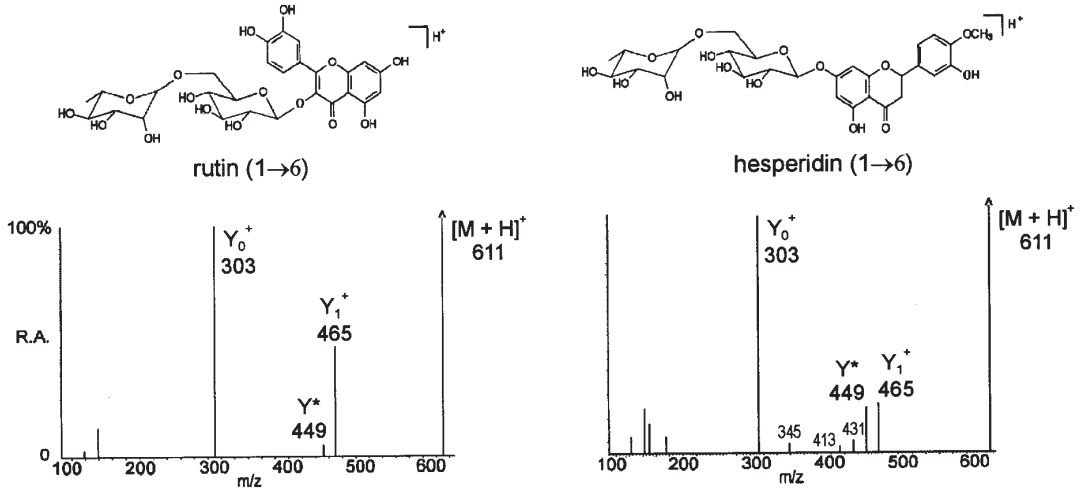


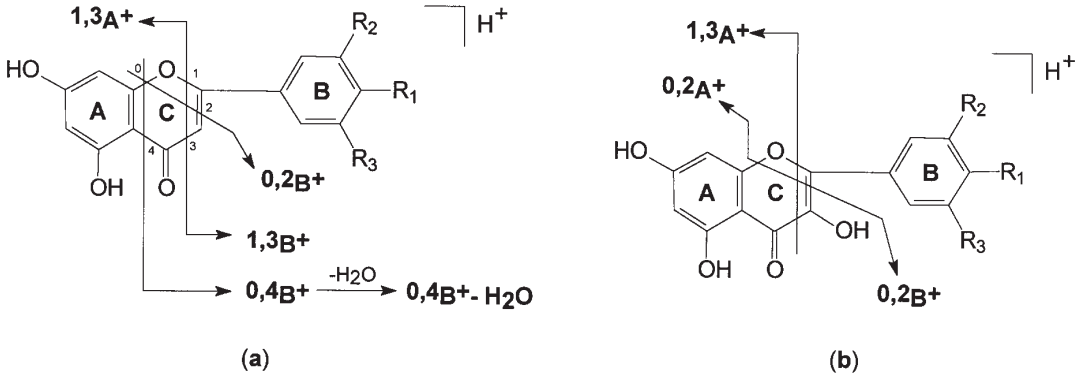
Figure 5. Low-energy CID spectra using helium as collision gas of protonated rutin (1→6) and hesperidin (1→6).

In conclusion, low-energy CID and metastable ion spectra of protonated flavonoid *O*-diglycosides can yield useful information on the carbohydrate sequence, the nature of the aglycone and the interglycosidic linkage type of isomeric rutinose [rhamnosyl-(α 1→6)-glucose] and neohesperidose [rhamnosyl-(α 1→2)-glucose] glycosides.

Concerning the structural elucidation of unknown flavonoid *O*-diglycosides, simple guidelines can be followed if resorting to metastable ion spectra and considering the relative abundances of the diagnostic ions Y_0^+, Y_1^+ and Y*. $Y_0^+ > Y_1^+$ points to an *O*-neohesperidoside (1→2 linkage), while $Y_0^+ < Y_1^+$ indicates an *O*-rutinoside (1→6 linkage). If in the case of $Y_0^+ > Y_1^+$ a Y* ion is present we are likely dealing with a flavanone-7-*O*-neohesperidoside. In the case of $Y_0^+ < Y_1^+$ a Y* ion can be present or absent. The absence of an Y* ion indicates a flavone-7-*O*-rutinoside. If the Y* ion is present and corresponds to the base peak, a flavanone-7-*O*-rutinoside is indicated while if it does not correspond to the base peak, a flavonol-3-*O*-rutinoside is pointed out.

A more detailed approach to the characterization of the aglycone part consists of selecting the Y_0^+ ion, which corresponds to the protonated aglycone part, and subjecting it to low-energy CID [8]. Low-energy CID spectra of Y_0^+ ions show simple fragmentation patterns, which allow characterization of the substituents in the A and B rings and, differentiation between flavones and flavonols. A more systematic ion nomenclature has been proposed that is conceptually similar to that introduced for the description of carbohydrate fragmentations in CID spectra of glycoconjugates (Scheme 2).

Figure 6 illustrates the low-energy CID spectra obtained for the Y_0^+ ion of (a) luteolin and (b) kaempferol. The most useful fragmentations in terms of aglycone identification are those that involve cleavage of two C-C bonds at positions 1/3, 0/2 and 0/4 of the C-ring, resulting in structurally informative $^{i,j}A^+$ and $^{i,j}B^+$ ions. This pair of product ions clearly provides the substitution pattern in the A and B rings of each compound and can be rationalized by retro Diels-Alder (RDA) reactions. $^{1,3}A^+$ and $^{0,2}B^+$ ions are common in all the spectra and a $^{1,3}B^+$ ion is found for all flavones, while $^{1,3}B^+ - 2H$ is characteristic for the flavonols. The additional hydroxyl group in position 3 of the flavonols results in more possibilities for fragmentation since their CID spectra are more complex than the spectra of the flavones. $^{0,2}A^+$, $^{0,2}A^+ - CO$, $^{1,4}A^+ + 2H$ and $^{1,3}A^+ - C_2H_2O$ ions are only seen



Scheme 2. Nomenclature and diagnostic product ions of protonated (a) flavones and (b) flavonols formed at low-energy CID [8].

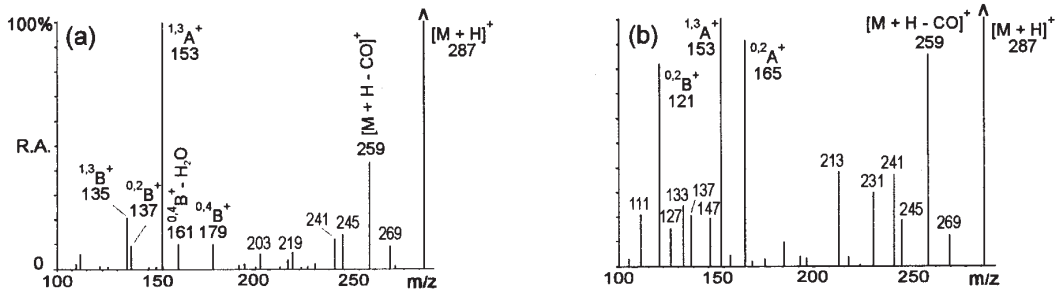


Figure 6. Low-energy CID spectra obtained for protonated (a) luteolin and (b) kaempferol.

for the flavonols, while $^{1,3}B^+$, $^{0,4}B^+$ and $^{0,4}B^+ - H_2O$ are only found for the flavones.

In addition to the $^{i,j}A^+$ and $^{i,j}B^+$ ions discussed, there are losses of small molecules and/or radicals from the $[M + H]^+$ ion. The loss of 18 u (H_2O), 28 u (CO), 42 u (C_2H_2O) and the successive loss of H_2O and CO is common for all compounds, while the flavones have additional losses of 68 u ($C_2H_2O - C_2H_2$) and the flavonols are more prone to loose 56 u ($2 \times CO$) and 18 u (H_2O) or 84 u ($C_4H_4O_2$) successively. Sometimes the loss of these smaller molecules can be useful for identifying the presence of specific functional groups, *i.e.* the presence of a methoxy group is easily detected by the loss of 15 u (CH_3) and 32 u (CH_3OH) from the $[M + H]^+$ precursor ion. This rather uncommon transition from an even-electron to an odd-electron ion is found to be characteristic of a phenolic methyl ether group.

Characterization of flavonoid *O*-diglycosides in the negative ion mode

As mentioned in the introduction we have started to evaluate MS/MS methods on deprotonated molecules with a view to apply MS/MS in combination with ES-LC-MS. Tests with rutin revealed that the best sensitivity in ES-LC-MS was obtained in the negative ion mode. Analogous findings have also been reported in other studies [14-17].

Figure 7 illustrates the metastable ion spectra of deprotonated naringin (1→2) and narirutin (1→6). In contrast to the positive ion mode, the relative abundance of Y_0^- and Y_1^- is the same for both compounds. It is worth noting that a $^{0,2}X_0^-$ ion can be seen with a relatively high abundance in the spectrum of the 1→2 isomer (neohesperidoside). This ion can be considered as characteristic of the 1→2 isomer, since it cannot be formed in the case of a 1→6 isomer (rutinoside) and consequently is absent for narirutin. The $^{0,2}X_0^-$ ion is formed by a retro [2 + 2 + 2] mechanism in the inner glucose residue and has been reported for kaempferol and naringenin 7-*O*-α-rhamnosyl (1→2)-β-glucosides [18].

The high-energy CID spectrum obtained for deprotonated naringin is given in figure 8. It can be noted that the sequence ion Y_1^- is a minor ion in the spectrum. In addition to the $^{0,2}X_0^-$ ion there is a second cross ring cleavage ion $^{1,5}X_0^-$ and ions characteristic of the aglycone part, naringenin, which could not be seen in the metastable ion spectrum (Fig. 7).

Further systematic studies will be undertaken in the future in order to establish whether the different fragmentation

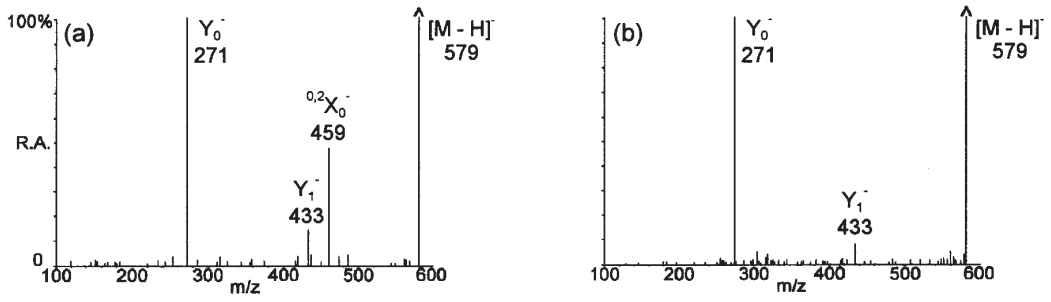


Figure 7. Metastable ion spectra of deprotonated (a) naringin (1→2) and (b) narirutin (1→6).

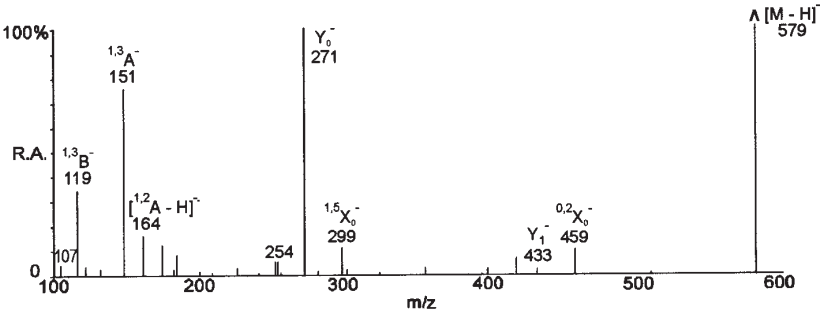


Figure 8. High-energy CID spectrum of deprotonated naringin.

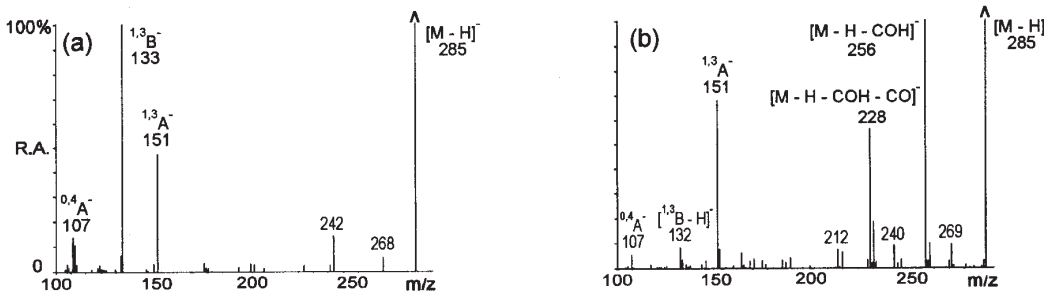


Figure 9. High-energy CID spectra obtained for deprotonated (a) luteolin and (b) kaempferol.

pattern found for the isomeric flavanone *O*-diglycosides, naringin and narirutin, also holds for other isomeric flavonoid *O*-diglycosides containing aglycones of the flavone and flavonol type.

A characterization of the aglycone part can also be achieved by performing a high-energy CID analysis on the Y_0^- ion. Figure 9 illustrates the high-energy CID spectra obtained for the deprotonated aglycone ion of (a) luteolin and (b) kaempferol. The fragmentation behaviour of the flavonoid aglycones in the negative ion mode is quite analogous to that in the positive mode. Cleavage of the C-ring by a retro Diels-Alder (RDA) mechanism leads to $^{i,j}A^-$ and $^{i,j}B^-$ ions, providing the substitution pattern in the A and B rings. $^{1,3}A^-$, $^{1,3}B^-$ and $^{0,4}A^-$ fragments are common for all compounds. The $^{i,j}A^-$ ions are more easily formed because of the relatively higher acidity of the 7-hydroxy group in comparison with the 4'-hydroxy group, *e.g.* $^{0,4}A^-$ is detected in the negative mode, whereas $^{0,4}B^+$ in positive. For flavonols containing two or more hydroxyl groups in the B ring, *i.e.* quercetin and myricetin, ions corresponding to $[^{1,2}A - H]^-$ and $[^{1,2}B + H]^-$ can be seen, while the spectra of the flavanones showed only $[^{1,2}A - H]^{-\bullet}$. Methylated compounds are characterized by the loss of 15 u resulting in a $[M - H - CH_3]^{-\bullet}$ base peak.

Conclusions

Both positive and negative tandem mass spectrometric techniques are useful in the structure elucidation of flavonoids. In our experience, we have found the positive mode to be more useful for structure elucidation of flavonoids and the negative mode to be more sensitive. *O*-, *C*- and *O*,*C*-diglycosides can be easily differentiated using first order FAB spectra or low-energy CID spectra. The protonated or deprotonated aglycone peak detected in the case of *O*-glycosides can be selected and subjected to low- or high-energy CID to allow the characterization of the substituents on the A and B rings and to differentiate between flavones, flavonols and flavanones. Both positive and negative ionization techniques provide information on the carbohydrate sequence, while metastable ion spectra and low-energy CID spectra obtained in the positive mode can be used to distinguish between the most common interglycosidic linkage types (1→2 and 1→6) encountered in flavonoid-*O*-glycosides.

Acknowledgments

We acknowledge the financial support by the Fund for Scientific Research (Belgium-Flanders; FWO; grant no. 6.0082.98) and the Concerted Actions of the Regional Government of Flanders (grant no. 99/3/34). F. Cuyckens thanks the "Vlaams Instituut voor de Bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie (IWT)" for a doctoral fellowship. M. Claeys is indebted to the FWO as a research director.

References

1. Harborne, J.B.; Mabry, T.J.; Mabry, H. *The flavonoids*; London: Chapman and Hall, 1988.
2. Harborne, J.B. *The flavonoids: Advances in research since 1986*; London: Chapman and Hall, 1994.
3. Markham, K.R. *Techniques of flavonoid identification*; London: Academic Press, 1982.
4. Amado, E.; Andersson, H.; Bardocz, S.; Serra, F. COST 916: Polyphenols in food; Proceedings of a European COST concerted action scientific workshop, Aberdeen, 1997.
5. Li, Q.M.; Van den Heuvel, H.; Delorenzo, O.; Corthout, J.; Pieters, L.A.C.; Vlietinck, A.J.; Claeys, M. *J. Chromatogr.* **1991**, *562*, 435-446.
6. Li, Q.M; Van den Heuvel, H.; Dillen, L.; Claeys, M. *Biol. Mass Spectrom.* **1992**, *21*, 213-221.
7. Li, Q.M.; Claeys, M. *Biol. Mass Spectrom.* **1994**, *23*, 406-416.
8. Ma, Y.L.; Li, Q.M.; Van den Heuvel, H.; Claeys, M. *Rapid. Commun. Mass Spectrom.* **1997**, *11*, 1357-1364.
9. Ma, Y.L.; Van den Heuvel, H.; Claeys, M. *Rapid. Commun. Mass Spectrom.* **1999**, *13*, 1932-1942.
10. Ma, Y.L.; Van den Heuvel, H.; Vedernikova, I.; Claeys, M. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 136-144.
11. Li, Q.M.; Dillen, L.; Claeys, M. *Biol. Mass Spectrom.* **1992**, *21*, 408-410.

12. Domon, B.; Costello, C. *Glycoconjugate J.* **1988**, *5*, 397-409.
13. Becchi, M.; Fraisse, D. *Biomed. Mass Spectrom.* **1989**, *18*, 122-130.
14. Swatsitang, P.; Tucker, G.; Robards, K.; Jardine, D. *Anal. Chim. Acta* **2000**, *417*, 231-240.
15. Careri, M.; Elviri, L.; Mangia, A. *Rapid. Commun. Mass Spectrom.* **1999**, *13*, 2399-2405.
16. Sägesser, M.; Deinzer, M. *J. Am. Soc. Brew. Chem.* **1996**, *54(3)*, 129-134.
17. Mauri, P.L.; Iemoli, L.; Gardana, C.; Riso, P.; Simonetti, P.; Porrini, M.; Pietta, P.G. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 924-931.
18. Denekamp, C.; Claeys, M.; Pocsfalvi, G. *Rapid. Commun. Mass Spectrom.* **2000**, *14*, 794-799.