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Differentiation of Interglycosidic Linkages in Permethylated Flavonoid Glycosides from Linked-Scan Mass Spectra (B/E)

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A series of per-*O*-methylated flavonoid di- and tri-glycosides, linked with 1−2 and/or 1−6 glycosidic bonds between sugar rings that were isolated from different plant materials were analyzed. It was demonstrated that the fragmentation behavior of permethylated flavonoid glycosides is dependent on the glycosidic bond placement between sugars. Y_n type fragment ions, created after glycosidic bond cleavage with oxygen retention on sugar at the reducing end for permethylated compounds, were observed in the normal and linked-scan mass spectra recorded for α (1−2) bonded conjugates of flavonoid di- and tri-glycosides. Moreover, for α (1−6) linked glycosides, Y* fragments created after rearrangement and elimination of internal sugar residues were observed in addition to Y_n type ions, but these fragment ions were not registered in normal desorption ionization spectra. This second type of fragmentation was also reported previously in collision-induced dissociation tandem mass spectrometry (CID MS/MS) spectra of some oligosaccharides and flavonoid glycosides, but their presence was independent of the glycosidic bonds placement between sugar rings.

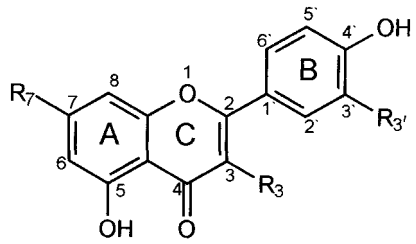
KEYWORDS: Flavonoid glycosides; desorption/ionization mass spectrometry; linked scan; permethylation; interglycosidic linkage

INTRODUCTION

Flavonoid conjugates constitute a very large group of plant secondary metabolites revealing diverse biological activities. This class of natural compounds plays an important role during development of plants and their interaction with the environment (*1*). They also affect human and animal health because of their significance in the diet, which is ascribed to their antioxidant properties (*2*), estrogenic action (*3*), and medicinal activities (*4*). Most flavonoids in plant tissue are present as mono-, di-, tri-, or tetra-glycosides. Different mass spectrometric techniques have been applied for identification and/or structural studies of this class of compounds (*5*).

When the mass spectrometric behavior of glycosylated flavonoids is considered, special emphasis has recently been given to the phenomenon of internal monosaccharide residue loss (*6*, *7*). This effect was first discussed in detail by Kovácik et al. (*8*−*10*). They examined desorption ionization collision-induced dissociation tandem mass spectra (CID MS/MS) of unmodified and per-*O*-methylated and *O*-acetylated oligosaccharides with different interglycosidic bonds. Claeys and co-workers reported that in low-energy CID MS/MS spectra of $[M+H]^+$ ions of unmodified flavonoid di-glycosides, possessing

Table 1. Flavonoids Glycosides Studied; Compounds Were Isolated from *Lupinus luteus* (**1**, **5**, and **6**), *Asclepias syriaca* (**2**), *Erysimum perofskiamum* (**3**), *Symphoricarpus albus* (**4**), *Malva neglecta* (**7**), and *Malva alcea* (**8**)



no	aglycone	R_3	R_3'	R_7
1	apigenin	H	H	*O*-Glc(1–2)Rha
2	kaempferol	*O*-Gal(1–2)Rha	H	OH
3	rhamnetin	*O*-Glc(1–2)Rha	OH	OCH_3
4	apigenin	H	H	*O*-Glc(1–6)Rha
5	kaempferol	*O*-Glc(1–6)Rha	H	OH
6	isorhamnetin	*O*-Glc(1–6)Rha	OCH_3	OH
7	kaempferol	*O*-Glc(1–2)[(1–6)Rha]Glc	H	OH
8	kaempferol	*O*-Glc(1–2)[(1–6)Rha]Xyl	H	OH

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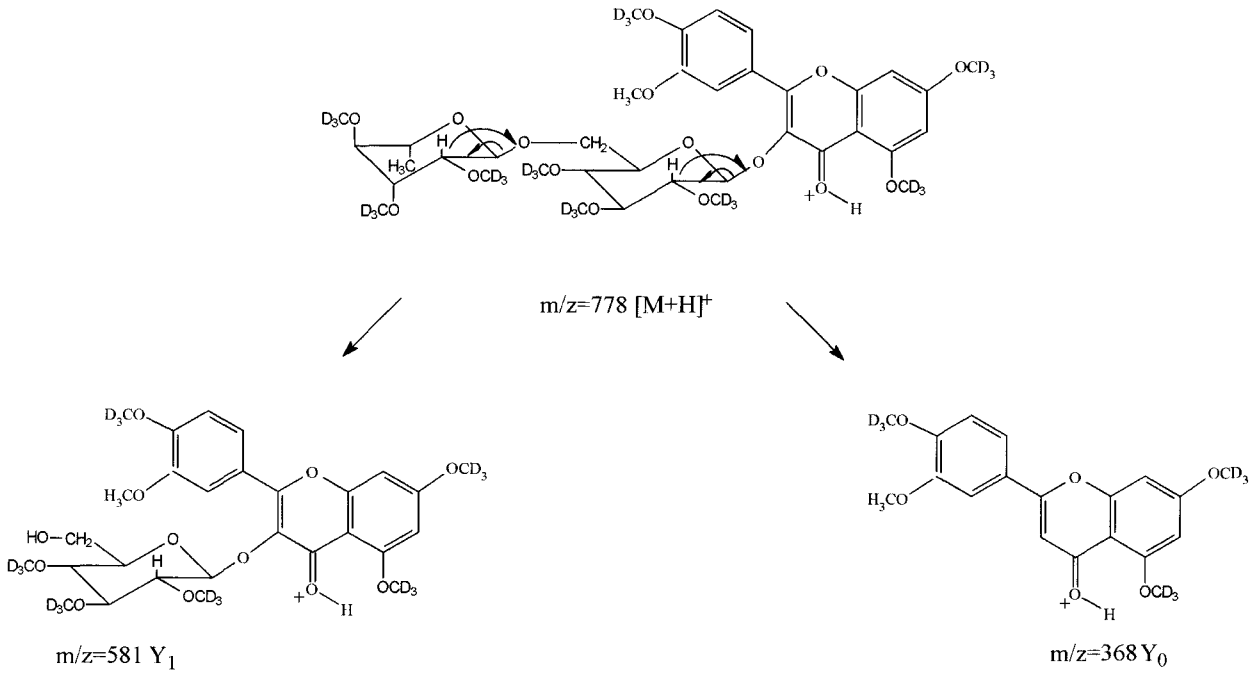
Table 2. Ions Registered in the LSIMS Mass Spectra: Normal and Linked-Scan (B/E = const) of Per-*O*-methylated Flavonoid Glycosides **1**–**8**

	$[M+H]^+$ *m/z* (%)		Y_1 *m/z* (%)		Y* *m/z* (%)		Y_0 *m/z* (%)	
no.	normal	linked-scan	normal	linked-scan	normal	linked-scan	normal	linked-scan
1	691 (58)	691 (100)	503 (2)	503 (3)	-	-	299 (100)	299 (15)
2	721 (10)	721 (100)	533 (5)	533 (2)	-	-	329 (100)	329 (3)
3	778 (15)[a]	778 (100)[a]	581 (10)[a]	581 (4)[a]	-	-	368 (100)[a]	368 (10)[a]
4	691 (10)	691 (100)	-	503 (1)	487 (2)	487 (2)	299 (100)	299 (20)
5	721 (70)	721 (100)	-	533 (19)	517 (6)	517 (28)	329 (100)	329 (10)
6	751 (23)	751 (100)	-	563 (4)	547 (4)	547 (25)	359 (100)	359 (28)
	778 (15)[a]	778 (100)[a]	-	581 (4)[a]	565 (3)[a]	565 (24)[a]	368 (100)[a]	368 (30)[a]
7	925 (2)	925 (100)	707 (3)-Glc 737 (1)-Rha	707 (3)-Glc 737 (4)-Rha	517 (5)	517 (25)	329 (100)	329 (10)
8	881 (10)	881 (100)	707 (5)-Xyl 693 (1)-Rha	707 (7)-Xyl 693 (4)-Rha	517 (15)	517 (35)	329 (100)	329 (10)

[a] Compound methylated with CD_3I.

Scheme 1. Proposed Mechanism for Y_n Type Ion Formation during Fragmentation of Protonated Per-*O*-methylated-d_3-Flavonoid Glycoside, **6** $[M+H]^+$



α (1−2) or α (1−6) glycosidic bonds, rearrangements leading to a loss of an internal sugar ring may occur (*6*). In the analyzed conjugates (flavone, flavonol, and flavanone di-glycosides) the structure of the aglycon and the place of disaccharide substitution on the aglycon influence the fragmentation pathway. Elimination of an internal sugar after rearrangement of rhamnose (Y* type fragment ion) was observed for α (1−2) and α (1−6) linked rhamnosyl di-glycosides (*6*). The presence or absence of these fragment ions was independent of the position of the glycosidic bond between the sugar rings in the flavonoid glycosides that were studied. From these investigations it was concluded that the information obtained from CID mass spectra of the unmodified compounds (presence of the Y* type fragment ions and the relative intensities) can be used to determine the glycosidic bond position between sugars in flavonol or flavanone di-saccharides (*6*, *7*).

In the earlier work on flavonoid oligosaccharides, it was demonstrated that derivatization improves sensitivity in MS analyses of carbohydrates and allows sequence and branching determinations to be made (*11*, *12*).

In our laboratory, different mass spectrometric techniques accompanied with chemical modifications of the compounds studied were applied for structural elucidation of flavonoid glycosides (*13*, *14*). This approach allowed us to differentiate various isomers of the flavonoid conjugates studied. We also reported increased sensitivity for the mass spectrometric analyses of permethylated compounds. Liquid secondary ion (LSI) mass spectra (normal and linked-scan) of unmodified and permethylated compounds have been performed. In this paper we demonstrate that the fragmentation pathway of permethylated di- and tri-glycosides of flavone (apigenin, **1**, **4**) and flavonols (kaempferol, rhamnetin, or isorhamnetin **2**, **3**, **5**−**8**) conjugates is dependent only on the glycosidic bond position between sugars, and that the structure of the aglycon has some influence on the relative intensities of fragment ions created after internal glucose rearrangement.

MATERIALS AND METHODS

Plant Material. Flavonoid glycosides were isolated from different plants belonging to six species: *Lupinus luteus* (Leguminosae), *Erysimum perofskiamum* (Cruciferae), *Symphoricarpus albus* (Caprifoliaceae), *Asclepias syriaca* (Asclepiadaceae), *Malva neglecta*, and *Malva alcea* (Malvaceae). According to the procedure described,

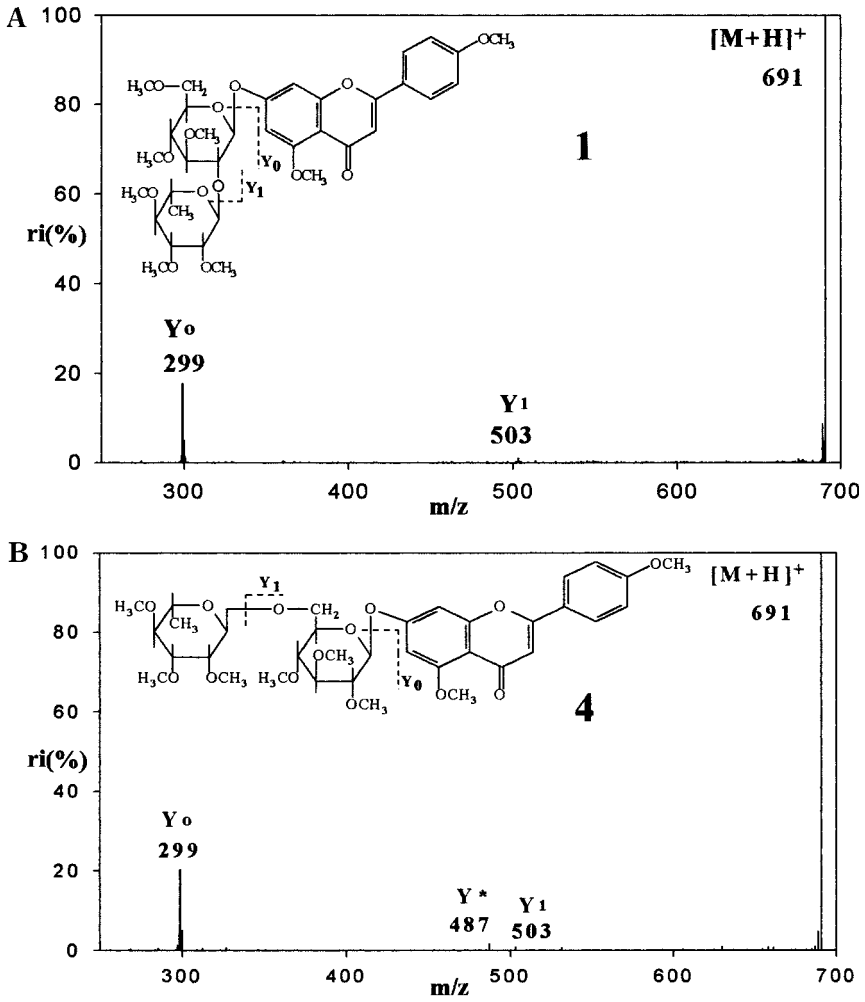


Figure 1. Linked-scan (*B*/*E* = const) mass spectra: (A) apigenin 7-*O*-(2′′-*O*-rhamnosyl) glucoside, **1**; and (B) apigenin 7-*O*-(6′′-*O*-rhamnosyl) glucoside, **4**.

structures of the compounds investigated were elucidated earlier on the basis of their UV, MS, and/or NMR spectra (*13*, *15*).

Methylation. Flavonoid glycosides were methylated with CH_3I or CD_3I in DMSO according to the modified Hakomori procedure (*17*).

Mass Spectrometry. Mass spectral analyses were performed on a double-focusing, reversed-geometry *B*/*E* mass spectrometer (AMD Intectra GmbH model 604, Harpstedt, Germany). The instrument was fitted with a cesium ion gun operating at an ion energy of about 12 keV and an accelerating voltage of 8 kV. Per-*O*-methylated flavonoid glycosides were dissolved exclusively in glycerol as the matrix. The linked-scan (*B*/*E* = const) mass spectra of product ions were recorded on the same instrument at a scan rate of 20 s per decade.

Theoretical Calculations. Theoretical calculations where performed with the MOPAC 6 program using the AM1 semiempirical method. For the purpose of simplifying calculations, the model compounds permethylated apigenin and permethylated kaempferol were chosen. Similar methodology was used by Claeys and co-workers (*6*) to elucidate the fragmentation pathways of unmodified flavonoid glycosides. The most stable conformations of the model compounds were found, and charges on oxygen atoms were calculated. The stabilities in terms of heat of formation for model compounds protonated at oxygen at C-5, oxygen at C-4, oxygen at C-3 (in the case of kaempferol), oxygen at C-7, oxygen at C-4′, and oxygen in position 1, were compared.

RESULTS AND DISCUSSION

In our studies we analyzed eight unmodified and per-*O*-methylated di- and tri-glycosides of flavones and flavonols isolated from different plant species, where aglycon moieties were *O*-glycosylated at C-7 or C-3 atoms (**Table 1**). The methylation procedure used in our investigation was not applicable for derivatization of flavanone glycosides (naringin and hesperidin), as it was found that during the derivatization procedure both aglycons underwent rearrangement to chalcones (data not presented). To describe the fragmentation patterns the nomenclature proposed by Domon and Costello (*17*) was used. The Y_n type fragment ions resulted from cleavage of one of the glycosidic bonds with the charge retained on the protonated fragment containing the aglycon. In the case of the Y_0 type fragment ions (protonated aglycons), the differences in the *m*/*z* values of the unmodified and permethylated compounds (**1**−**8**) enabled elucidation of glycosylation pattern and determination of sugar rings distribution on the aglycons. In addition, in the mass spectra of 1−6 linked flavonoid di-saccharides, Y* type fragment ions were observed in the normal and linked-scan mass spectra. This fragmentation results from rhamnose rearrangement and elimination of the internal glucose residue as described earlier for unmodified oligosaccharides and flavonoid di-glycosides with 1−2 or 1−6 glycosidic linkages (*6*).

In the studied flavonoid glycosides, the bonds between rhamnose and glucose were α (1−6) or α (1−2). These are two of the most common linkages between sugars in glycosylated flavonoid conjugates of plant origin (*18*). The positions of linkages between sugars for compounds **1**−**8** were determined by mass spectrometric approaches, where methanolysis products of permethylated flavonoid glycosides were acetylated and

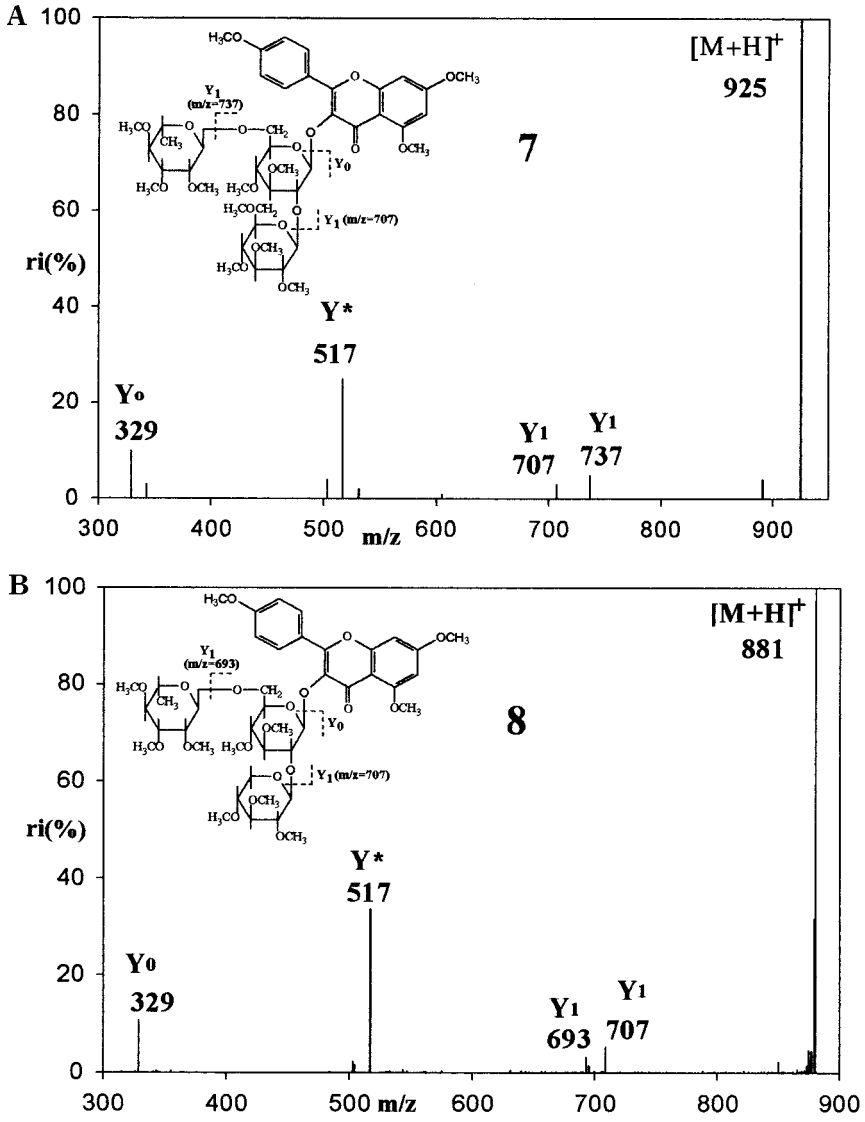


Figure 2. Linked-scan (*B*/*E*) mass spectra: (A) kaempferol 3-*O*-[(6″-*O*-rhamnosyl) 2″-*O*-glucosyl] glucoside, **7**; and (B) kaempferol 3-*O*-[(6″-*O*-rhamnosyl) 2″-*O*-xylosyl] glucoside, **8**.

analyzed with GC/MS. The 2- and/or 6-acetylated tetra- or tri-*O*-methylated glucose and appropriate aglycons were identified according to the retention times and fragmentation pathways elucidated from the mass spectra (*13*). In the paper on the structural elucidation of flavonoid glycosides, Y* type ions were not classified properly in the mass spectra of di-glycosides **5** and **6**, which were identified in yellow lupin (*13*). These fragment ions were described as Z type ions created following cleavage of the glycosidic linkage with retention of the oxygen atom on the sugar ring at the nonreducing end.

In the positive ion mode, LSI mass spectra of all permethylated flavonoid glycosides **1**−**8** showed intense Y_0 fragment ions (protonated aglycon) with 100% relative intensity (**Table 2**). The Y_1 fragments created after cleavage of the glycosidic bond between the sugar rings had very low intensities (S/N ratio, maximum 6:1), and they were observed only for compounds **1**−**3** with the α (1−2) linkage. In the branched tri-glycosides **7** and **8**, both of the possible Y_1 fragment ions were observed with low abundance after cleavage of 1−2 or 1−6 linked sugars. The rupture of the bond between C‴-1 and C″-2 carbons of sugar rings was more pronounced. However, for α (1−6) bonded di-glycosides **4**−**6**, Y_1 fragment ions were observed only in the linked-scan spectra.

The mechanism of fragmentation leading to creation of Y_0 and Y_1 ions in permethylated derivatives was different from that proposed by Claeys and co-workers for the unmodified flavonoid glycosides (*17*, *19*). In per-*O*-methylated compounds, transfer of a hydrogen atom came from the C-2″ sugar carbon atom instead of the C-2″ hydroxyl group during elimination of the rhamnose ring or rhamnosyl glucoside. This mechanism was confirmed by analyses of mass spectra for the natural products permethylated with CD_3I or CH_3I (compounds **3** and **6**). In both cases, only transfer of the hydrogen atom to create Y_0 and Y_1 fragment ions was observed (**Table 2** and **Scheme 1**).

In the normal and linked-scan mass spectra of permethylated compounds (**4**−**8**) with the α 1−6 linkage between rhamnose and glucose, Y* type fragment ions were observed after elimination of internal sugar (compounds **4**−**6**) or 1−2 linked disaccharide (compounds **7** and **8**) and rearrangement of the rhamnose ring to the aglycon. The neutral fragment created during rearrangement was anhydroglucose for **4**−**6** or glycosyl anhydroglucoside for **7** and **8**. The S/N ratios of these ions were low in the normal mass spectra, but in the linked-scan experiments (*B*/*E* = const), Y* type fragment ions had over 20% of the intensities relative to that of the parent ions in the spectra of flavonol glycosides **5**−**8**. The only exception was

Table 3. Partial Charges on Selected Oxygen Atoms of Permethylated Kaempferol and Apigenin Calculated by the AM1 Method

	charge for permethylated compound	
atom	kaempferol	apigenin
O-1	–0.13	–0.14
O at C-4	–0.28	–0.29
O at C-5	–0.17	–0.17
O at C-7	–0.20	–0.20
O at C-4′	–0.21	–0.21
O at C-3	–0.21	

apigenin rutinoside **4**, where the intensity of this ion was low and comparable with that in the normal LSI mass spectra (**Figures 1** and **2**). In the permethylated flavone and flavonol di- and tri-glycosides with the α 1−6 bond between rhamnose and glucose (**4**−**8**), the structures of the flavonoid and the placement of sugars on the aglycon had no direct influence on the presence of Y* type ions in the mass spectra. On the other hand, the differences in the relative intensities of these fragment ions in the linked-scan mass spectra of 3-*O*-glycosylated flavonols and 7-*O*-glycosylated flavone were essential (**Table 2**).

Claeys and co-workers analyzed FAB CID low energy MS/MS spectra of unmodified flavone, flavanone, and flavonol neohesperosides α (1−2) and rutinosides α (1−6) (*6*, *7*). The authors did not observe Y* type fragment ions in the mass spectra of 7-*O*-substituted apigenin (flavone) and kaempferol (flavonol) di-glycosides, linked with one of the above-mentioned bonds between the sugars. The rhamnose ring rearrangement and elimination of internal glucose was observed only for α (1−2) and α (1−6) linked di-saccharides of 7-*O*-substituted flavanones and 3-*O*-linked flavonols. Moreover, in the mass spectra of α (1−6) linked kaempferol 7-*O*-rhamnosyl glucoside (flavonol conjugate) a very weak peak was also observed. In the CID MS/MS or metastable ion spectra registered for flavanone and flavonol di-glycosides, the relative intensities of Y* type ions were dependent on the aglycon structure, as well as on the position of the flavonoid hydroxyl substituted with disaccharide, the linkage position between sugars, and the collision energy. According to these authors, the structure of the C ring in the flavonoid molecule and presence of the hydroxyl group at C-5 affects the possible mobility of the proton attached to the C-4 carbonyl oxygen in the aglycon moiety of the unmodified glycoside molecule during fragmentation. This influences the efficiency of sugar ring rearrangement and possible elimination of internal glucose in α (1−2) and α (1−6) linked unmodified flavonoid di-glycosides (*6*, *7*).

During our studies we found that, for permethylated glycosides, the rearrangement of rhamnose occurred only for α (1−6) linked di-saccharides. The Y* fragment ions were not observed in the mass spectra of compounds with the α (1−2) linkage. Theoretical calculations performed on permethylated flavone (apigenin) and flavonol (kaempferol) demonstrated that the most energetically favorable site for protonation is the carbonyl oxygen in the C ring of the aglycon moiety. The largest negative charge in the model compounds was localized on the carbonyl oxygen at C-4 of the C ring, indicating that the protonated molecule had the highest stability when the proton was on the C-4 oxygen (**Table 3**). In permethylated apigenin, the proton attached to the oxygen at C-4 is situated between the O atom at C-4 and the O atom at C-5. However, for derivatized kaempferol there were two possible places of proton substitution: between the O atom at C-4 and the O atom at C-5. or between the O atom at C-4 and the O atom at C-3. Both of these structures were possible and they had similar stabilities. Protonation of the oxygen in position 1 of the C ring was also considered, but the calculations showed that such a protonated molecule is much less stable than one in which the proton is on the carbonyl oxygen as mentioned above (**Table 4**). From calculations it was clear that the negative charge on

Scheme 2. Proposed Mechanism for Y* Type Ions Formation during Fragmentation of Protonated Per-*O*-methylated Flavonoid Glycoside, **5** $[M+H]^+$

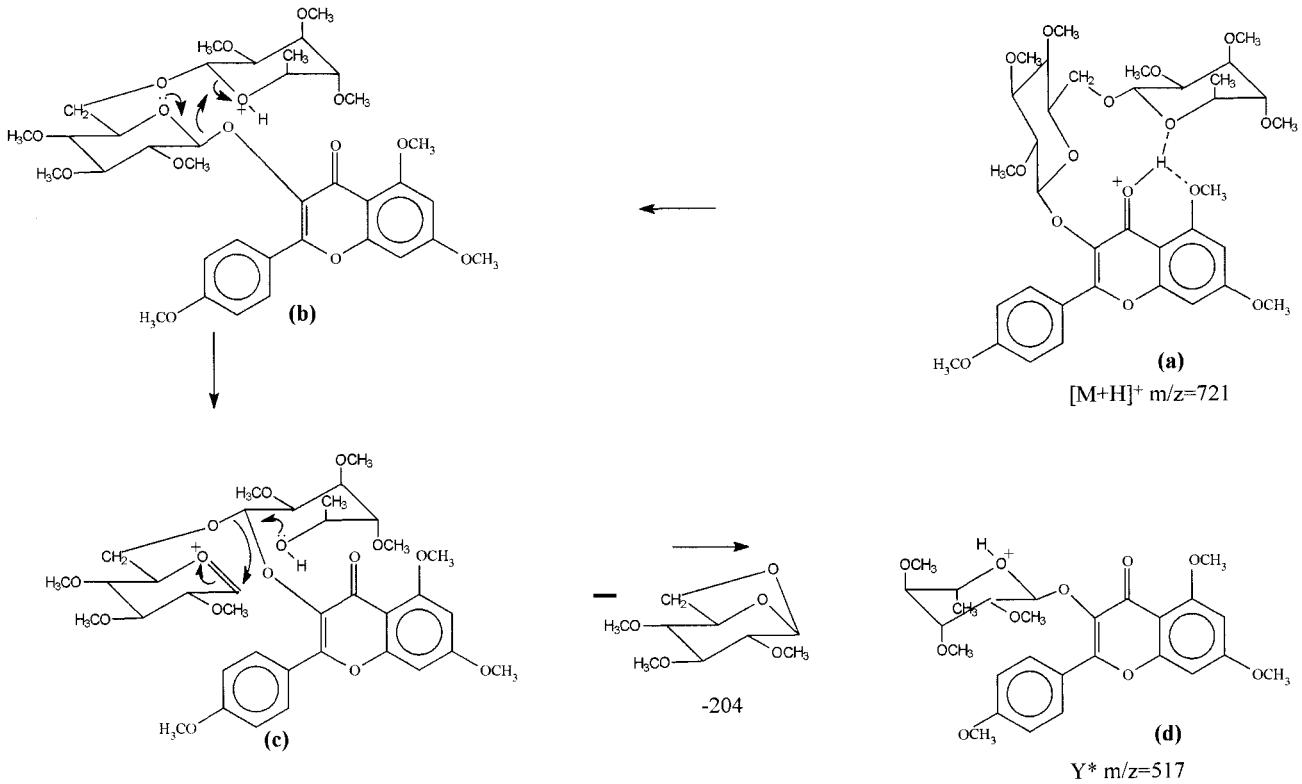


Table 4. Heats of Formation of Cations Derived from Protonation of Permethylated Kaempferol and Apigenin

	heat of formation for permethylated compound	
protonation site	kaempferol kcal/mol	apigenin kcal/mol
O-1	55.8	88.24
O at C-4 (between O at C-4 and O at C-5)	1.43	32.0
O at C-4 (between O at C-4 and O at C-3)	0.98	
O at C-7	55.36	87.86
O at C-4′	52.1	86.56

oxygen 1 is much lower than that at the C-4 carbonyl oxygen (**Table 3**). As can be seen, the starting point for the fragmentation pathway should be the permethylated flavonoid glycoside protonated between the oxygen at C-4 and the oxygen at C-5. Because of methylation of all hydroxyl groups, creation of hydrogen bonds in the protonated molecules of glycosides was possible only between a hydrogen bonded to the C-4 carbonyl and the C-5 oxygen of the methoxyl group in flavone (**1**, **4**) and C-3 or C-5 oxygen of 3-*O*-glycosylated flavonols (**2**, **3**, and **5**−**8**). From a model it is clear that steric hindrance resulting from the presence of methyl groups on the oxygens precludes free rotation around the glycosidic bonds in the protonated molecules $[M+H]^+$ of permethylated flavonoid glycosides. However, the ion conformation permitting transfer of the proton from the C-4 carbonyl to the hemiacetal oxygen of the rhamnose ring is possible only for isomers **4**−**8**, where the glycosidic bond between glucose and rhamnose was α (1−6) (**Scheme 2**).

An additional proof for the exclusive rearrangement of an α (1−6) linked sugar (rhamnose) in permethylated di- and triglycosides was obtained after mass spectral analysis of kaempferol conjugates: 3-*O*-[(2″-*O*-glucosyl) 6″-*O*-rhamnosyl] glucoside (**7**) and 3-*O*-[(2″-*O*-xylosyl) 6″-*O*-rhamnosyl] glucoside (**8**) (see **Table 2** and **Figure 2**). In their normal and linked-scan mass spectra, intense Y* type fragment ions (over 5% and 25% relative intensities, respectively) were observed. These ions were created after elimination of di-glycoside: 1−2 linked glucosyl−glucoside **7** or 1−2 linked xylosyl−glucoside **8** following rearrangement of rhamnose to the aglycon.

The methylation of all hydroxyl groups in the flavonoid di- and tri-glycosides changed the conformation of the molecules. This affected the proton rearrangement in $[M+H]^+$ ion leading to formation of Y* fragment ions, due to the difference in the possibilities of the proton transfer from the C-4 carbonyl oxygen to the hemiacetal oxygen of the rhamnose in the di-saccharide substituted at C-7 of flavone or C-3 of flavonol. On the basis of the data obtained from linked-scan spectra at B/E = const, we suggest that differentiation of α (1−2) and α (1−6) linkages between sugars rings in per-*O*-methylated flavone and flavonol glycosides is possible. The complete inhibition of the rhamnose ring rearrangement to the aglycon in the derivatized α (1−2) isomeric flavonoid di-glycosides was observed. However, the abundance of Y* type ions is dependent on the substitution position of diglycoside on the aglycon. The distance of the hemiacetal oxygen of rhamnose residue to the protonated carbonyl at the C-4 position in the C ring influences the proton rearrangement efficiency. The comparison of our results obtained for methylated flavonoid glycosides with those obtained for unmodified compounds studied with CID MS/MS techniques (*6*) reveals differences in the occurrence of rhamnose rearrangement in the case of flavone 7-*O*-diglycosides However, this phenomenon was not observed at all in the spectra of both isomers of the unmodified compounds. For methylated flavonol 3-*O*-rutinosides (with α (1−6) linkage) the rearrangement was registered (abundant Y* fragment ions in linked mass spectra) and for 3-*O*-neohesperosides (with α (1−2) linkage) this fragmentation was not observed. In unmodified flavonol 3-*O*-disaccharides the rearrangement was registered in the mass spectra of both isomers, but the abundance of the fragment ions was lower for α 1−2 isomers (*6*).

Flavonoid and isoflavonoid glycosides, due to different structural modifications of the aglycon such as hydroxylation, methylation and glycosylation pattern, reveal a significant structural diversity. For this reason some structural data from the mass spectra registered with desorption ionization for underivatized compounds are not accessible. Simple chemical modifications of flavone, isoflavone, and flavonol glycosides, prior to structural elucidation of this group of natural products with mass spectrometric techniques, permits an increase in the amount of structural information about the structure of aglycons or sugars and glycosidic bonds placement obtainable from the mass spectra (*12*, *13*). Analysis of linked mass spectra of the methylated flavonoid glycosides additionally allows enlargement of the range of accessible structural information about the position of the glycosidic bonds between sugars for unknown flavonoid glycosides.

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