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# Application of mass spectrometry to structural identification of flavonoid monoglycosides isolated from shoot of lupin (*Lupinus luteus* L.)*

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**Flavonoid glycosides constitute important group of plant secondary metabolites. This class of natural products play significant role in different physiological processes. A new methodological approach where mass spectrometric techniques are applied to structural studies of this class of compounds is presented. Four flavonoid O-monoglycosides and one C-monoglycoside were isolated from green parts of lupin (*Lupinus luteus* L.). Several different mass spectrometric techniques were applied to structural elucidation of isolated compounds. Desorption ionization mass spectrometry was used for registration of mass spectra of intact and derivatized (permethylated) flavonoid glycosides. In some cases electron impact mass spectra of permethylated compounds were also recorded. Methylated samples after methanolysis and further derivatization of free hydroxyl groups (methylation or acetylation) were analyzed with gas chromatography-mass spectrometry. Combined information drawn from the registered mass spectra enabled us to define molecular mass, structure of aglycones and sugars, and positions of glycosidic bonds on the aglycon. Structures of four flavonoid monoglycosides were elucidated as follows: genistein 7-*O*-glucoside (1), genistein 4′-*O*-glucoside (2), 2′-hydroxygenistein 7-*O*-glucoside (3), and apigenin**

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**Abbreviations:** MS, mass spectrometry; GC/MS, gas chromatography-mass spectrometry; *m/z*, mass to charge ratio; FAB, fast atom bombardment; LSIMS, liquid secondary ion mass spectrometry; DCI, direct chemical ionization; EI, electron impact; $M^{+\bullet}$, molecular ion; $[M+H]^+$, protonated molecule; [M–H], deprotonated molecule; NBA, nitrobenzyl alcohol; RDA, retro Diels-Alder reaction; MIKES, mass analyzed ion kinetic energy spectrum.

**or genistein 8-*C*-glycoside (5). For the fourth O-glycoside (4) only molecular mass and masses of the aglycone and sugar were estimated.**

Flavonoid glycosides constitute a structurally diverse group of plant secondary metabolites [1]. The flavonoids are usually divided into many subclasses: flavones, isoflavones, flavanones, anthocyanins, aurones and others. These natural products play a very important role in plant development and physiology, especially during their interactions with other living organisms. Flavonoid glycosides and free aglycones are involved in pathogenic and symbiotic interactions with microorganisms [2, 3]. They affect also plant–plant and plant–insects interactions [4]. This class of compounds is used as a phytochemical marker in chemotaxonomic studies [5] and also has antioxidant and UV protective properties [6, 7].

One of the physico-chemical methods, characterized by a very high sensitivity, and commonly applied to structural analysis of natural products is mass spectrometry. Different ionization methods were used for registration of mass spectra of pure flavonoid glycosides [8]. Glycosides are thermally labile, polar and non-volatile compounds and their derivatization is required when electron impact ionization is applied. The development of new desorption techniques: fast atom bombardment (FAB) [9], liquid secondary ion mass spectrometry (LSIMS) [10], direct chemical ionization (DCI) [11] has allowed the analysis of these secondary metabolites without the necessity of chemical derivatization. Also collision-induced decomposition tandem mass spectrometry (CID MS/MS) in association with desorption ionization methods was applied to structural studies of flavonoid glycosides [12–16]. In order to improve the signal-to-noise ratio of protonated molecules $[M+H]^+$ during desorption ionization, permethylation of flavonoid glycosides was performed prior to the registration of mass spectra [15].

From normal desorption mass spectra and CID mass spectra of unknown flavonoid glycosides it is possible to estimate the molecular mass, size of the aglycone moiety and sugar or sugars in the molecule of the compounds studied. On the other hand, gas chromatography-mass spectrometry (GS/MS) of chemically modified compounds was used for the elucidation of the exact structure of the aglycone and confirmation of sugar identity, also substitution position of sugars on the aglycone in O-glycosides [15–17].

In our paper we demonstrate a methodological approach where different mass spectrometric techniques and chemical modifications are applied to structural evaluation of four flavonoid O-glycosides **1–4** and one C-glycoside **5** isolated from green parts of lupin (*Lupinus luteus* L.) cv. Ventus. The structural analysis of compounds was performed on submilligram quantities of samples.

## MATERIALS AND METHODS

### Chemicals and reagents

All solvents employed were analytical grade (POCh, Poland), and additionally distilled before use. Thin-layer chromatography (TLC) silica gel H60 $F_{254}$ plates and normal and reversed phase silica gels ($C_{18}$ and silanized) utilized for column chromatography were purchased from Merck (Germany). Standard of sugars (glucose and galactose) were from Sigma (U.S.A.), genistein and 2′-hydroxygenistein were isolated from white lupin roots during earlier investigations, as described previously [18, 19].

### Plant material, isolation of flavonoid glycosides

Yellow lupin seeds (*Lupinus luteus* L.) cv. Ventus were sterilized with 1.5% (v/v) hypochlorite solution for 20 min and washed 10

times with sterile water. They were sown in autoclaved perlite and grown in greenhouse conditions for three weeks. Plants were collected and divided at the stem base. Roughly 320 g of aerial parts were cut and ground with a mortar and pestle. The homogenate was extracted with 1500 ml of 80% aqueous methanol in an ultrasonic bath for 30 min. The extract was filtered through a Büchner funnel and the residual material washed with 200 ml of methanol. Combined filtrates were concentrated at 40°C under reduced pressure to the aqueous residue.

The extracted lupin secondary metabolites were further partitioned using sequential extraction with organic solvents: hexane, ethyl acetate, and n-butanol saturated with water. Combined ethyl acetate and butanol extracts were concentrated under reduced pressure to an oily residue. In the first step of purification, column chromatography on silica gel H60 (Merck) in a step gradient of methanol in chloroform (from 0% to 70% of MeOH) was used. Fractions containing compounds of interest were combined. Further purification was achieved on a column with silanized silica gel in a gradient of methanol in water (from 15% to 60% of MeOH). Five flavonoid glycosides were finally purified on silica gel TLC plates developed with phase B (see further). Samples were additionally desalted on RP $C_{18}$ cartridges. Compounds were loaded on the columns in 0.01 M HCl and eluted with 50% MeOH in water. All chromatographic separations were monitored by thin-layer chromatography on silica gel with UV detection at 254 nm, and color reaction with Gibbs reagent [20]. TLC plates were developed either in: $CHCl_3/MeOH/H_2O$ = 40:10:1, by vol. (Phase A) or $EtOAc/MeOH/H_2O$/conc. $NH_3$ = 60:15: 15:1, by vol. (Phase B).

### Derivatization procedures

***Methylation.*** Methylation reactions of standards (isoflavonoids and sugars) and intact flavonoid glycosides or products of their methanolysis were performed according to the modified Hakomori procedure in dimethylsulfoxide ($Me_2SO$) with methyl iodide [21]. In a tightly capped vial the sample (about 100 $\mu$g) was dissolved in 0.5 ml of $Me_2SO$ and 20 mg of fine powdered dry KOH was added. The reaction was started with the injection of 50 $\mu$l of methyl iodide and performed for five minutes at room temperature with agitation. After this time 1 ml of water was added and the methylated product extracted with chloroform.

***Methanolysis.*** 1 M HCl in MeOH was prepared from dry, ice-cold methanol and acetyl chloride. An appropriate volume of acetyl chloride was added to 5 ml of MeOH. Methanolysis of methylated or unmodified flavonoid glycosides was performed in screw-capped vials in 0.5 ml of 1 M HCl in MeOH, for 2 h at 100°C. After evaporation of the solvent in a stream of dry $N_2$, samples were subjected to further steps of the derivatization procedure.

***Acetylation.*** Acetylation of permethylated and methanolyzed flavonoid glycosides was done in a mixture of pyridine/acetic anhydride (1:1, v/v), overnight at room temperature. Acetylated samples were used directly for GC/MS analyses.

### Mass spectrometric techniques

***Liquid secondary mass spectrometry.*** Mass spectral analyses were performed on AMD Intectra (Germany) double focusing, reversed geometry B/E mass spectrometer Model 604, which was fitted with a cesium ion gun operating at ion energy of about 12 keV, the accelerating voltage was 8 kV. Samples were dissolved in different matrices (glycerol, thioglycerol, nitrobenzyl alcohol, triethanolamine) and analyzed in positive and negative ion mode. The collision-induced decomposition product ion spectra in the linked scan B/E mode, were performed with helium as collision gas in the cell, in the first field free region at such pressure, that the intensity of

the ion beam was reduced by 50% of its value. CID mass spectra were recorded at the scan rate of 20 s/decade.

***Electron impact ionization mass spectrometry (EI MS).*** EI mass spectra were recorded on an AMD Intectra (Germany) double focusing, reversed geometry B/E mass spectrometer Model 402 operating at ionization voltage of 70 V and source temperature 200°C, samples were evaporated from metal capillaries introduced to the source of mass spectrometer. The collision-induced decomposition product ion spectra in the linked scan B/E mode, were recorded as described above.

***Gas chromatography-mass spectrometry.*** Samples were subjected to GC/MS analyses on a Hewlett-Packard Gas Chromatograph model 5890II with Mass Selective Detector model 5971A. The instrument was equipped with XTI-5 (Restek Co., U.S.A.) fused silica capillary column (0.25 mm × 30 m). The carrier gas was helium at a flow rate 1 ml/min. The column temperature was programmed from 140°C (held for 2 min) at 5°C/min to 300°C, which was maintained for 5 min. The injector temperature was 250°C. Mass spectra were recorded in the range of 50–650 u.

## RESULTS AND DISCUSSION

Different mass spectrometric techniques were applied to structural elucidation of five flavonoid O- or C-monoglycosides **1–5** isolated from aerial parts of yellow lupin plants (Fig. 1). Structures of three O-glucosides **1–3** were established, for the C-glycoside **5** and the O-glycoside **4** only partial structural information was obtained.

For complete structural characterization of flavonoid glycosides, the following information has to be established: molecular weight, an aglycone structure, sugar or sugars identity, differentiation between O- and C-glycosides, glycosylation position of aglycone, or linkages between sugars. In order to obtain the above mentioned data appropriate MS techniques have to be used. Molecular weight information can be obtained from LSIMS mass spectra of unmodified or methylated compounds. In the case of monoglycosides the same information is amenable from EI mass spectra of methylated flavonoid glycosides. From LSIMS spectra is also possible to estimate the size of the aglycone and sugar (hexose or deoxyhexose) moieties in the case of O-glycosides due to fragment ions created after cleavage of glycosidic bonds. GC/MS analyses of methanolysis and methylation products obtained from O-glycosides permit precise estimation of aglycone and sugar structures. The glycosylation position of the aglycone in O-glycosides may be established after GC/MS analysis of sample obtained after methanolysis followed by acetylation of permethylated compound. Unumbigouse differentiation between O- and C-glycosides is possible after registration of CID LSIMS linked scan spectra of product ions for unmodified and/or methylated flavonoid glycosides. An overview of mass spectrometric techniques applicable in structural studies of flavonoid glycosides is presented in Scheme 1.

For registration of mass spectra of unmodified and permethylated flavonoid monoglycosides **1–5** desorption ionization (LSIMS) was used, in some cases for derivatized compounds, electron impact ionization (EI) was also applied. LSIMS CID linked scan mass spectra in the B/E mode of unmodified and methylated compounds were recorded. Permethylated flavonoid O-glycosides **1–4** after additional chemical treatment were further analyzed with GC/MS. These analyses permitted to elucidate flavonoid or isoflavonoid structure of the aglycone and substitution position of the sugar moiety on the aglycone core, also structure of the sugar rings was also confirmed.

For the flavonoid O-glycoside **4**, from the desorption ionization LSIMS normal and CID linked scan mass spectra molecular weight of compound **4**, sizes of the flavonoid aglycone

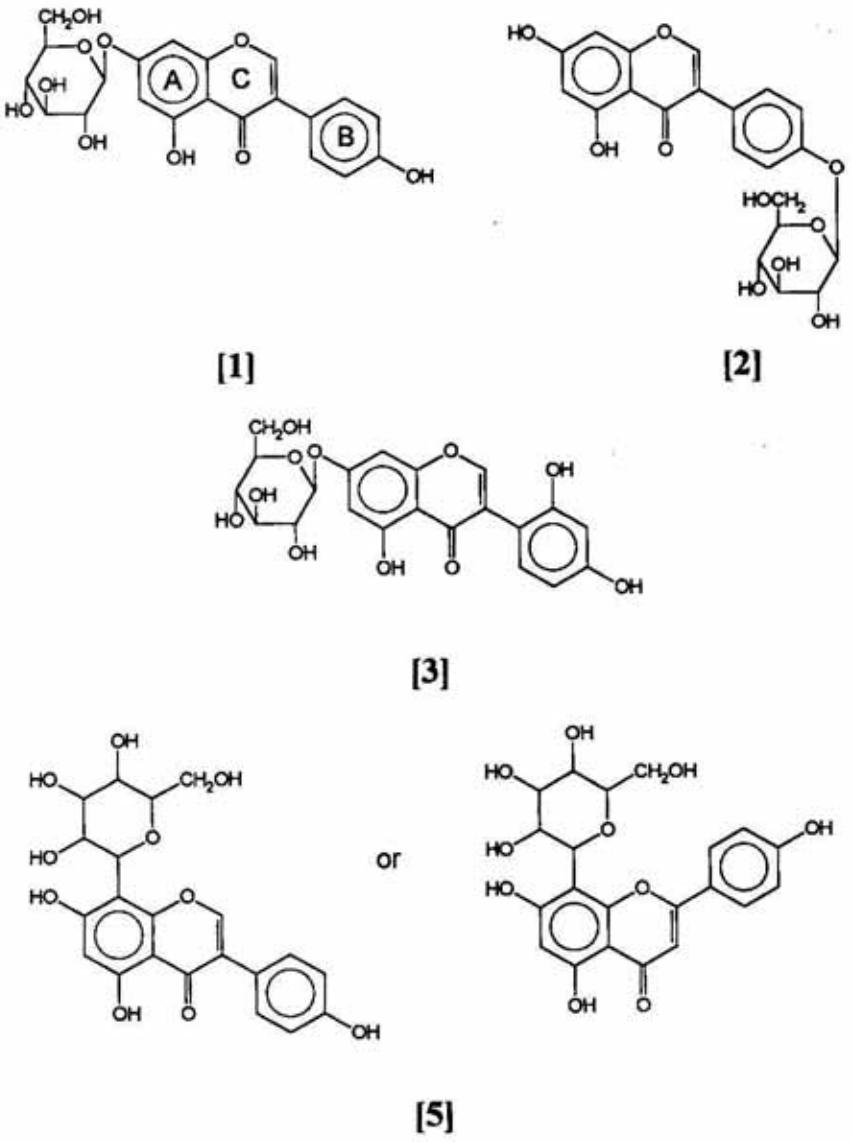
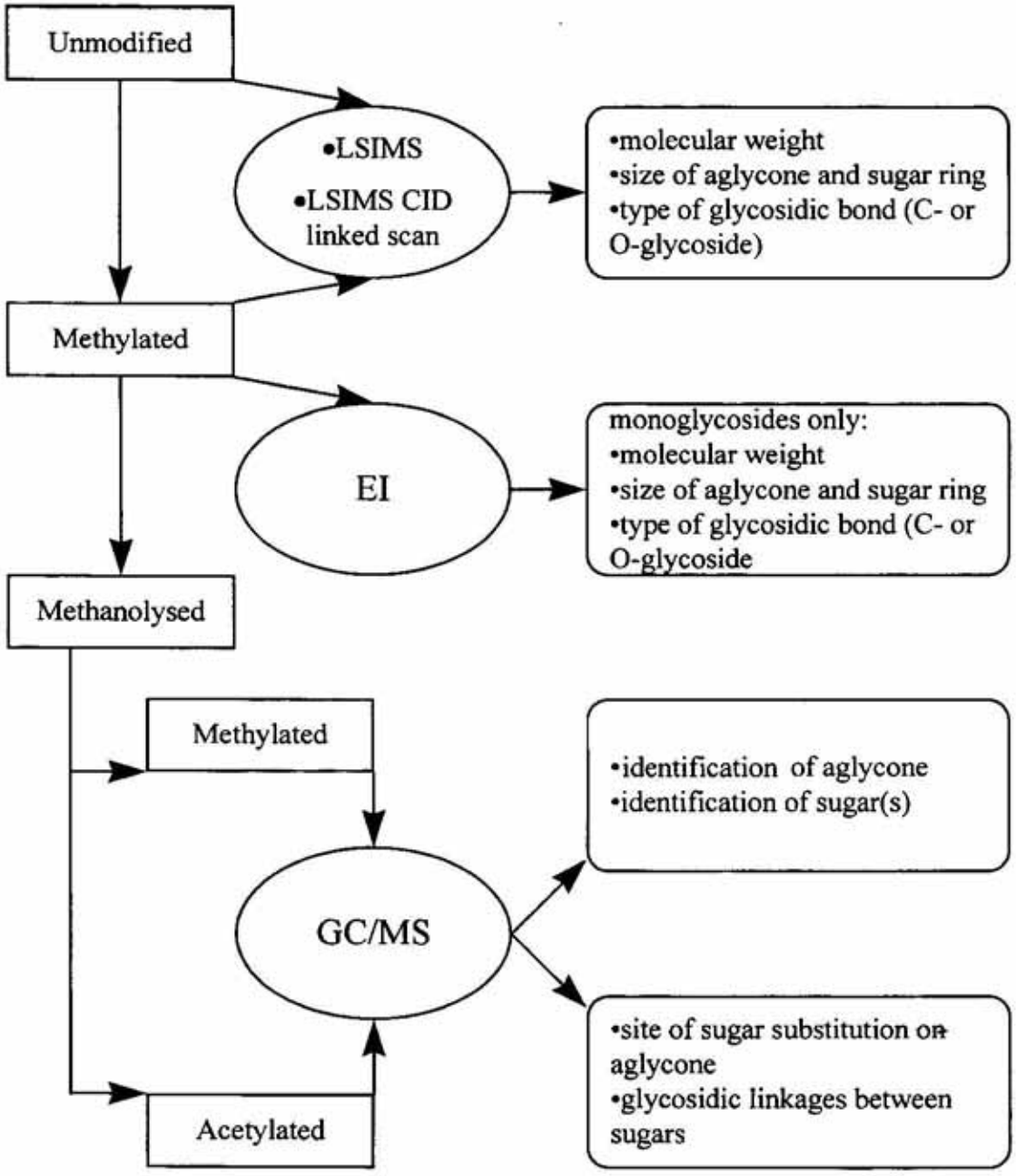



**Figure 1. Structures of identified flavonoid glycosides isolated from shoot of yellow lupin: genistein-7-*O*-glucoside (1), genistein-4′-*O*-glucoside (2), 2′-hydroxygenistein-7-*O*-glucoside (3), genistein or apigenin-*C*-8-glucoside (5).**

and sugar were estimated. Results obtained after GC/MS analyses permitted only for final identification of the sugar as glucose. Also for the C-glycoside **5** a complete evaluation of structure was not possible. Determination of the structural type of aglycone (flavone or isoflavone) from GC/MS data obtained after hydrolysis in hydriodic acid [22] and methylation of products was not possible due to degradation of compound **5** during chemical treatment.

**Liquid secondary ion mass spectra**

Nitrobenzyl alcohol (NBA) or glycerol were chosen as liquid matrices for registration LSIMS mass spectra in positive or negative ion mode of unmodified and permethylated compounds. During analysis of unmodified compounds, in order to improve solubility of the samples, 0.5 $\mu$l of $Me_2SO$ was added to the matrix. In both matrices, it was possible to maintain long lasting signal of protonated




**Scheme 1.**

$[M+H]^+$ or deprotonated $[M-H]^-$ molecules, this was particularly important for the registration of collision-induced decomposition linked scan spectra of product ion in B/E mode. Best results were obtained when NBA was used as matrix, both in positive and negative ion mode (Tables 1 and 2, Figs. 2–4). Only mass spectra of permethylated compound **3** were recorded in glycerol as the matrix, because of the possible interference of NBA cluster ions $[2NBA+Na]^+$ at 329 *m/z* with fragment ions $[A+H]^+$, created after cleavage of glycosidic bond, where the charge is retained at the aglycone moiety.

Intensities of sample ions in the mass spectra were influenced by the presence of impuri-

ties, both organic compounds and sodium cations. For these reasons desalting of samples on RP $C_{18}$ cartridges improved the quality of the obtained LSIMS mass spectra. In the mass spectra of unmodified O-glycosides **1–3** registered in the positive ion mode, apart from $[M+H]^+$, strong $[M+Na]^+$ ions were also observed, with signal-to-noise ratio for protonated $[M+H]^+$ and sodiated $[M+Na]^+$ molecules not better than 10:1. Only for compound **4** this ratio was much lower (about 4:1). The same signal-to-noise ratio was observed for fragment ions $[A+H]^+$ created after rupture of glycosidic bond.

In the positive ion LSIMS mass spectrum of the C-glucoside **5**, the only signal observed was one originating from the protonated molecule $[M+H]^+$ at 433 *m/z*. On the other hand, in negative ion mass spectra of unmodified compound **5**, ions of deprotonated molecule $[M-H]^-$ at 431 *m/z* and strong fragment ions at 311 *m/z* and 297 *m/z*, generated after rupture of sugar moiety $[M-H-120]^-$, and $[M-H-134]^-$, respectively (Table 1), were observed.

In the mass spectra of permethylated compounds **1–4** signal-to-noise ratio of protonated molecules $[M+H]^+$ was higher than 30:1, but the main ions were fragment ions $[A+H]^+$, created after cleavage of O-glycosidic bonds (Table 1 and Fig. 2b). This fragmentation pathway was not observed in the mass

**Table 1. Positive and negative ions LSIMS mass spectra of unmodified and permethylated compounds 1–4**

| Compound | | $[M+H]^{+}$ [a] $[M-H]^{-}$ [b] *m/z* (% ri) | $[A+2H]^{+}$ [a] $[A-H]^{-}$ [b] *m/z* (% ri) | Other ions *m/z* (% ri) |
|---|---|---|---|---|
| Genistein-7-*O*-glucopyranoside | unmodified (+)[a] | 433 (60%) | 271 (100%) | 455 (65%)-$[M+Na]^+$ |
| | (−)[b] | 431 (100%) | 269 (90%) | |
| **1** | methylated (+)[a] | 517 (58%) | 299 (100%) | 539 (18%)-$[M+Na]^+$ |
| Genistein-4′-*O*-glucopyranoside | unmodified (+)[a] | 433 (100%) | 271 (15%) | 455 (80%)-$[M+Na]^+$ |
| | (−)[b] | 431 (100%) | ND | |
| **2** | methylated (+)[a] | 517 (100%) | 299 (55%) | 539 (35%)-$[M+Na]^+$ |
| 2′-Hydroxygenistein-7-*O*-glucopyranoside | unmodified (+)[a] | 449 (100%) | 287 (70%) | 471 (25%)-$[M+Na]^+$ |
| | (−)[b] | 447 (50%) | 285 (100%) | |
| **3** | methylated (+)[a] | 547 (80%) | 329 (100%) | 569 (18%)-$[M+Na]^+$ |
| Tetrahydroxyflavon-*O*-glucopyranoside | unmodified (+)[a,c] | | | |
| | (−)[b] | 447 (70%) | 285 (100%) | |
| **4** | methylated (+)[a] | 547 (100%) | 329 (80%) | |
| Genistein- or apigenin-8-*C*-glycopyranoside | unmodified (+)[a] | 433 (100%) | ND | ND |
| | (−)[b] | 431 (80%) | 269 (7%) | 311 (100%), 297 (15%) |
| **5** | methylated (+)[a] | 531 (100%) | ND | 553 (20%)-$[M+Na]^+$, 369 (30%), 355 (40%), 341 (20%) |

[a]Positive ion spectra; [b]negative ion spectra; [c]mass spectrum not registered; ND, ion not detected.

**Table 2. Product ions registered in LSIMS CID linked scan mass spectra of unmodified and permethylated compounds 1–4**

| Compound | | $[M+H]^+$ / $[M-H]^-$ *m/z* (% ri) | $Y_0^+$ *m/z* (% ri) | Other ions *m/z* (% ri) |
|---|---|---|---|---|
| Genistein-7-*O*-glucopyranoside | unmodified[b,c] | 433 (100%)<br>431 (100%) | 271 (14%)<br>269 (24%) | |
| **1** | methylated[b] | 517 (90%) | 299 (100%) | |
| Genistein-4′-*O*-glucopyranoside | unmodified[b] | 433 (100%) | 271 (35%) | |
| **2** | methylated[a] | | | |
| 2′-Hydroxygenistein-7-*O*-glucopyranoside | unmodified[a] | | | |
| **3** | methylated[b] | 547 (100%) | 329 (10%) | |
| Tetrahydroxyflavon-*O*-glycopyranoside | unmodified[a] | | | |
| **4** | methylated[b] | 547 (100%) | 329 (20%) | |
| Genistein- or apigenin-8-*C*-glycopyranoside | unmodified[c] | 431 (100%) | – | 297 (5%), 311 (45%), 341 (2%) |
| **5** | methylated[b] | 531 (100%) | – | 369 (3%), 355 (10%), 341 (2%) |

[a]LSIMS/CAD linked scan mass spectrum not registered; [b]positive ion LSIMS/CAD linked scan mass spectrum in B/E mode; [c]negative ion LSIMS/CAD linked scan mass spectrum in B/E mode; % ri, percentage of relative intensity; $Y_0^+$, fragment ion created after cleavage of glycosidic bond.

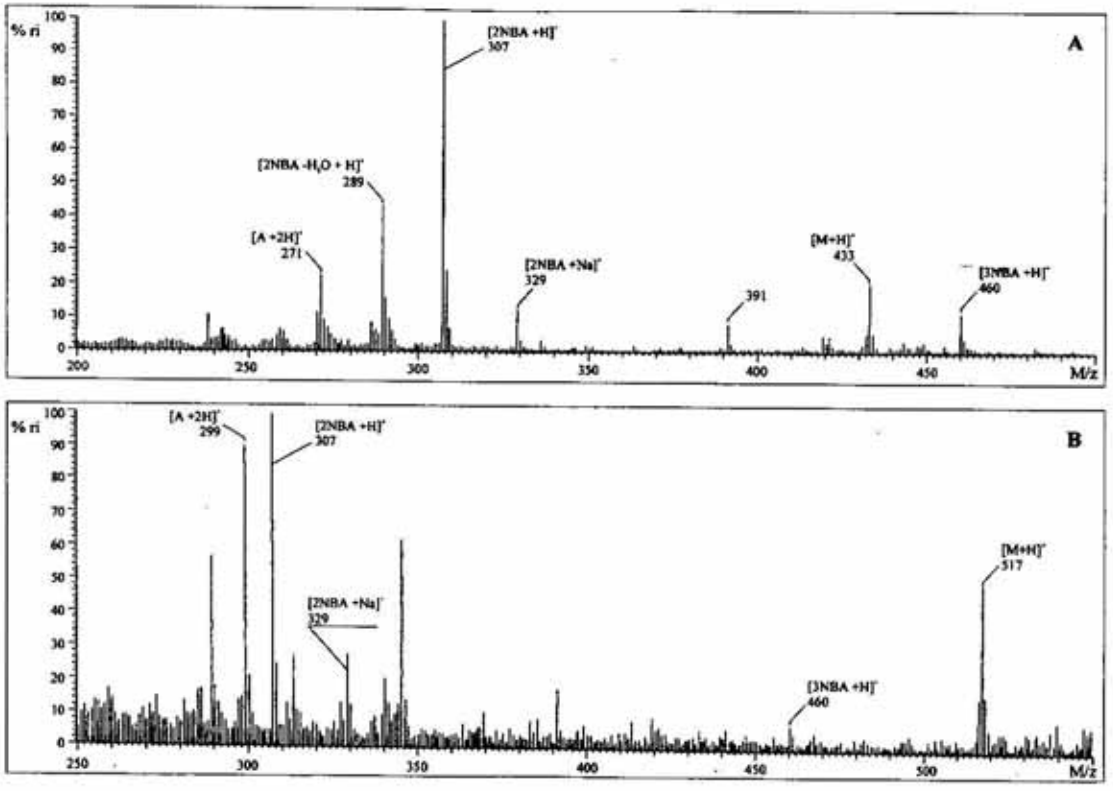



**Figure 2. LSIMS mass spectrum of compound 1.**

**A,** unmodified, positive ions; **B,** permethylated, positive ions.

spectrum of methylated C-glycoside **5**, where fragment ions originated from cleavage of bonds in sugar ring at 369, 355 and 341 *m/z* (see Table 1) were registered.

It was not possible to differentiate between O- and C-glycosides only on the basis of *m/z* value for $[M+H]^+$ ions of unmodified compounds due to the same molecular weight. However, permethylation of flavonoid glycosides enabled this type of identification as the difference of 14 atomic mass units for protonated molecules was observed due to methylation of the additional free hydroxyl group in the aglycone moiety of C-glycoside **5** (Table 1).

The presence of $[A+H]^+$ fragment ions created after cleavage of glycosidic bonds in compounds **1–4** was confirmed by collision induced decomposition linked scan product ions spectra of protonated molecule $[M+H]^+$ or deprotonated molecule $[M-H]^-$ recorded in B/E mode. Good quality CID linked scan mass spectra were obtained for unmodified and permethylated compounds in positive or negative ion mode (Table 2 and Figs. 3, 4).

According to the data obtained by Becchi and Fraisse from FAB metastable ion kinetic energy spectra (MIKES) and FAB CID/MIKES spectra of isovitexin (C-6 apigenin monoglucoside) and vitexin (C-8 apigenin monoglucoside) [13], a substantial difference in the ratio of relative intensities of metastable ions created after degradation of the glucose ring was observed. This ratio for the ions $[M-H-120]^-$ and $[M-H-90]^-$ was high (about 5:1) in the case of vitexin, but in MIKES spectrum of isovitexin it was lower than 2:1 [13]. This difference in the ratios of the intensities of the above mentioned metastable ions allows one to draw a conclusion about the site of sugar substitution on the aglycone. During analyses of the C-glycoside **5**, CID linked scan spectra of product ions were registered for unmodified and permethylated compound in the

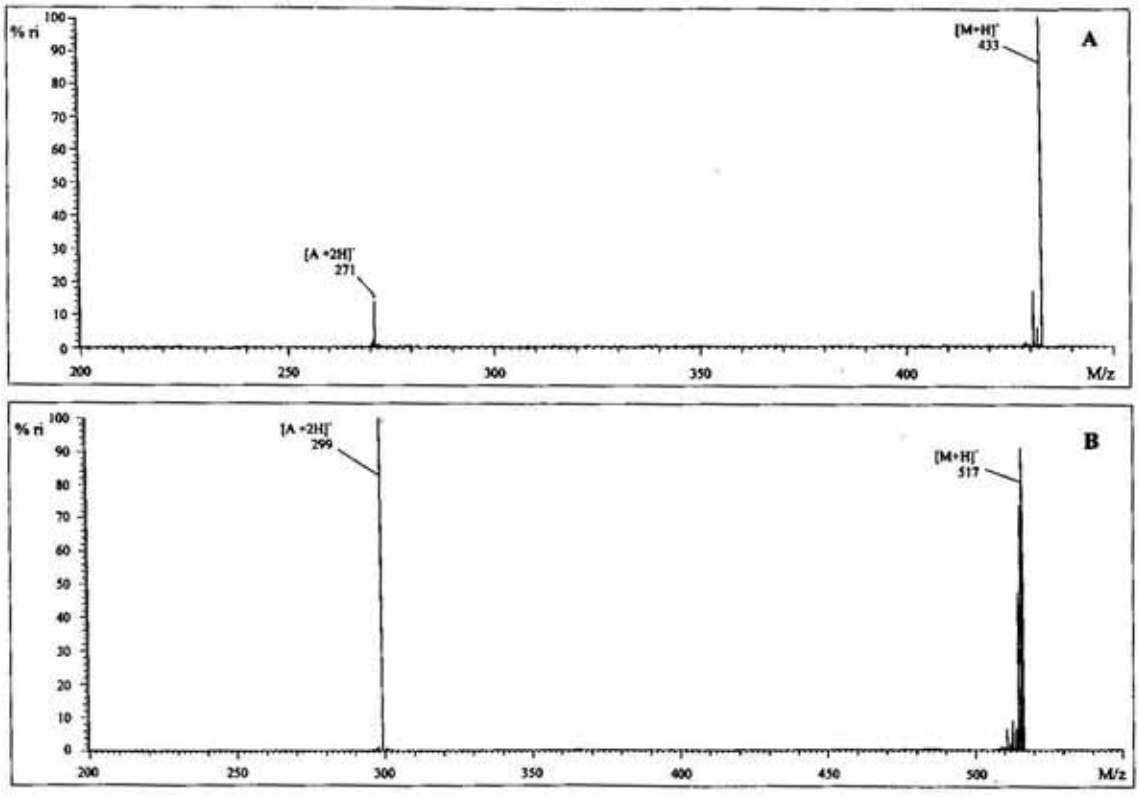



**Figure 3. LSIMS CID linked scan mass spectra of compound 1.**

**A**, unmodified, positive ions; **B**, permethylated, positive ions.

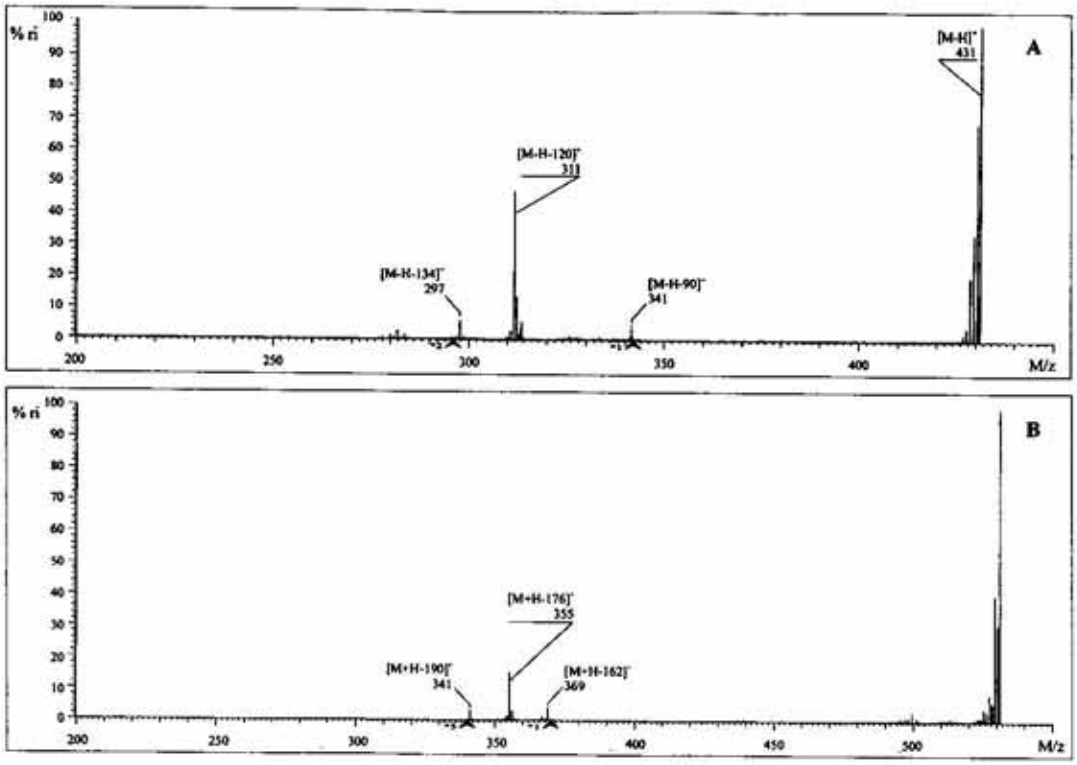



**Figure 4. LSIMS CID linked scan mass spectra of compound 5.**

**A,** unmodified, negative ions; **B,** permethylated, positive ions.

negative and positive ion mode, respectively. It was possible to determine the position of glucose substitution on the flavonoid core at carbon C-8, on the basis of the ratios of the product ions intensities in the linked scan spectrum (Figs. 4a and 5a). For unmodified compounds intense ions $[M-H-120]^-$ at 311 *m/z* and small ones at 341 *m/z* $[M-H-90]^-$ and 297 *m/z* $[M-H-134]^-$ were observed. The calculated ratio of intensities of product ions at 311 *m/z* and 341 *m/z* was consistent with this observed for C-8 apigenin glucoside in FAB CID/MIKES spectrum registered by Becchi & Fraisse [13]. A fragmentation pathway was proposed for methylated derivative **5** after linked scan analyses; structures of ions at 355, 369 and 341 *m/z*, created after cleavage of bonds in the sugar ring, are shown in Fig. 5b.

From normal and linked scan LSIMS mass spectra of unmodified and permethylated compounds **1–5** it was possible to estimate molecular mass, number of hydroxyl groups substituted on the aglycone moieties and size of sugar rings. Additionally, in the case of the C-glycoside **5**, substitution position on the aglycone was estimated.

### Electron impact mass spectra

Electron impact mass spectra of permethylated compounds **3** and **5** were recorded. According to literature data, relative intensities of ions in the mass spectra of permethylated C-6 and C-8 monoglycosides of apigenin differ substantially [23]. The main difference was the presence of $[M-31]^+$ fragment ion created after elimination of methoxyl group in the spectrum of apigenin C-6 glucoside, and higher relative intensity (86%) of molecular ion in the case of C-8 isomer. In EI mass spectrum of flavonoid C-glycoside isolated from lupin shoots, the fragment ion $[M-31]^+$ was not registered and the intensity of the molecular ion was over 50% of relative intensity of the main ion in the spectrum. On the basis of

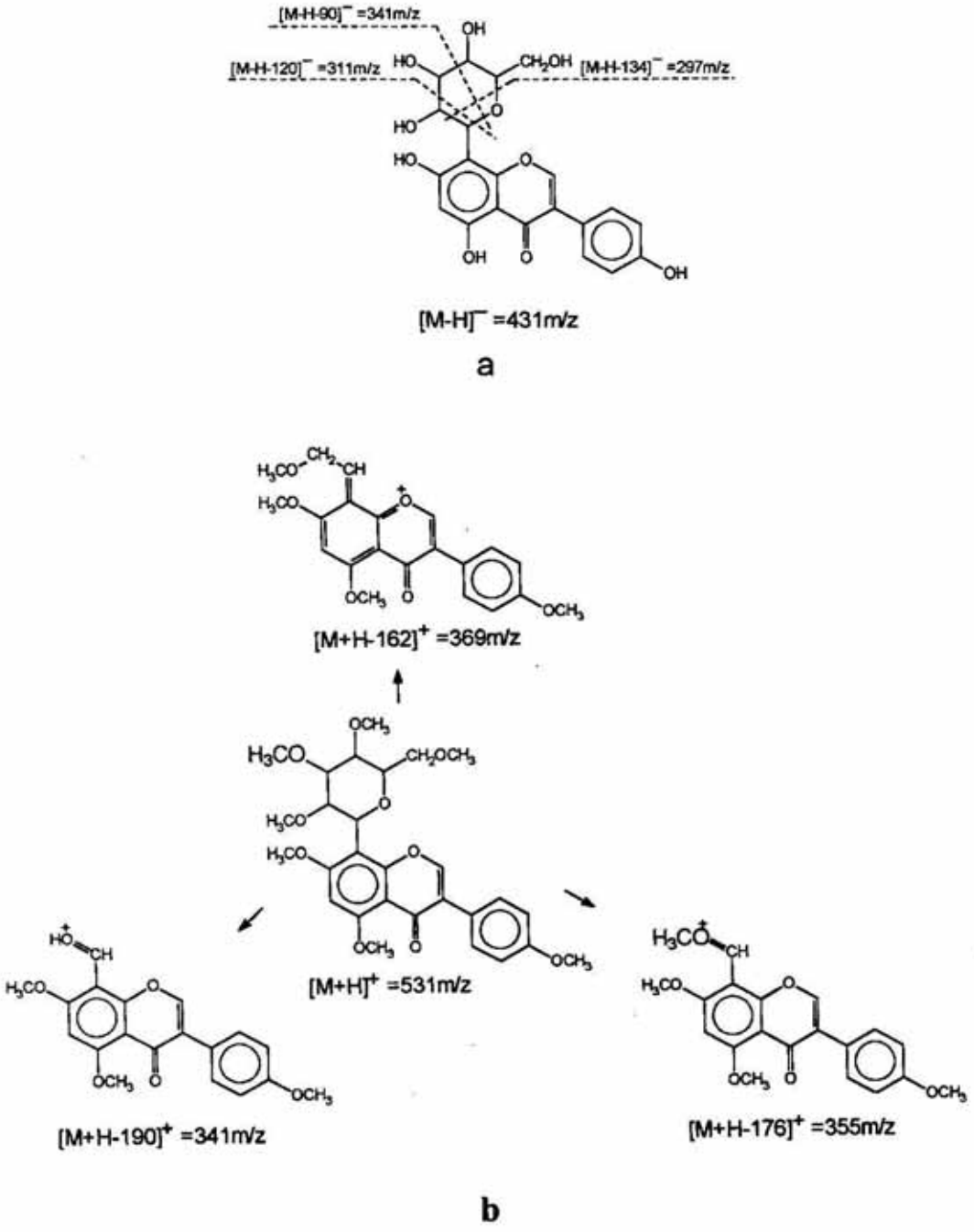



**Figure 5. Fragmentation pathway of compound 5 based on CID linked scan product ions spectra: unmodified compound (a), methylated compound (b).**

these data it was possible to conclude that substitution of hexose in this compound was at C-8 carbon of the aglycone. This information was consistent with earlier information obtained from CID linked scan spectra.

The EI spectrum of permethylated compound **3** revealed strong molecular ion at 546 *m/z* and fragment ions created after glycosidic bond cleavage with charge retention on the aglycone moiety at 328 *m/z*. Also a series of intense ions characteristic for the fragmentation of hexose bound to aglycone were recorded at 218, 187, 155 and 111 *m/z* [24]. However, fragment ions created after RDA re-

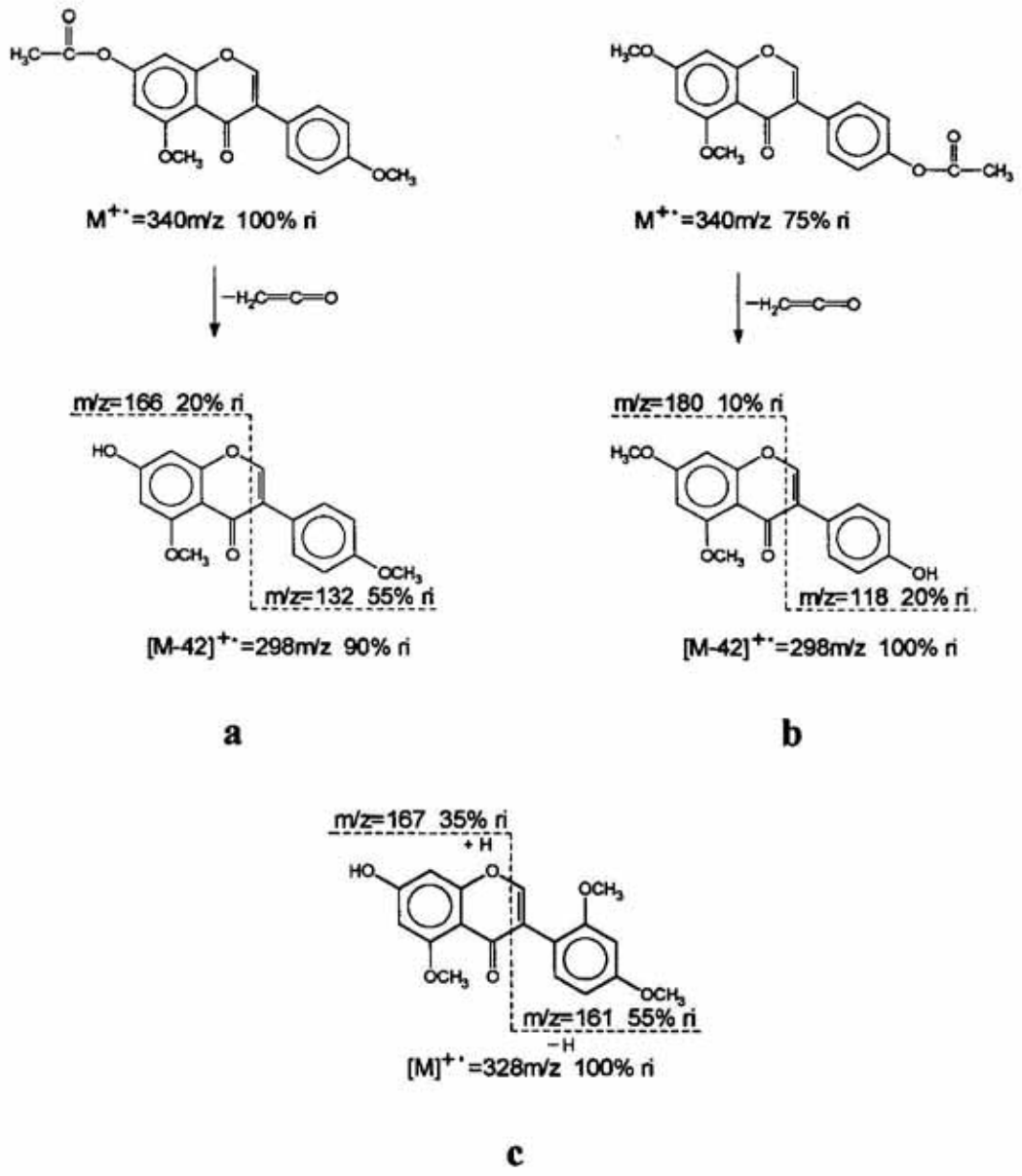



**Figure 6. Fragmentation pathway of aglycones obtained after methylation, methanolysis and acetylation of compounds 1–2, compound 3 was not subjected to acetylation reaction: 7-*O*-acetyl-4′,5-di-*O*-methylgenistein (a), 4′-*O*-acetyl-5,7-di-*O*-methylgenistein (b), 2′,4′,5-tri-*O*-methyl-2′-hydroxygenistein (c).**

action, occurring in the C ring of isoflavone core in the aglycone part of the molecule were not univocally recognized in the mass spectrum.

### Gas chromatography/mass spectrometry

For the elucidation of sugar identity and aglycone structure, gas chromatography/mass spectrometric analysis of chemically modified compounds **1–4** was used. For confirmation of aglycones and sugars structure, analyses of methylated, methanolysed and again methylated, samples of flavonoid glycosides were studied; GC/MS of methylated standards of isoflavones and sugars was also performed. In order to obtain additional information about the site of sugar substitution on the aglycone molecule, permethylated fla-

**Table 3. Aglycones and sugars identified after GC/MS analyses of chemically modified (methanolyzed and methylated) flavonoid O-glycosides 1-4**

| Standards | $t_R$ | MW | Flavonoid glycosides | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| 4',5,7-Tri-*O*-methyl genistein | 29.87 | 312 | + | + | - | - |
| 2',4',5,7-Tetra-*O*-methyl 2'-hydroxygenistein | 31.06 | 342 | - | - | + | - |
| Tetra-*O*-methoxyflavon | 31.52 | 342 | - | - | - | + |
| 1,2,3,4,6-Penta-*O*-methyl-glucopyranoside | 4.05/4.64 | 250 | + | + | + | + |

$t_R$, retention time.

vonoid O-glucosides **1–3** were hydrolyzed in 1 M HCl methanol solution, and products of methanolysis were further acetylated. Analysis of permethylated compounds allowed, on the basis of retention time and mass spectra, to define structures of isoflavonoid aglycones and sugars. Aglycones in compounds **1** and **2** were identified as genistein, and in glycoside **3** as 2'-hydroxygenistein. For all three compounds, glucose was recognized as the sugar bound to the isoflavonoid moiety (Table 3). GC/MS analyses of products obtained after permethylation, methanolysis and acetylation of compounds **1–2**, and permethylation and methanolysis of glycoside **3**, allowed the identification of the position of sugar ring substitution to hydroxyl group at isoflavonoid skeleton (Table 4 and Fig. 6). Glucose was substituted to C-7 or C-4' hydroxyl group in compound **1** and **2**, respectively. The third compound was defined as 2'-hydroxygenistein 7-*O*-glucoside. Acetylation position of isoflavone aglycones obtained from glucosides **1–2** was elucidated from mass spectra. Masses of fragment ions created after retro Diels-Alder (RDA) reaction in C ring of 7-O- or 4'-O-acetylated genistein differed substantially, indicating the site of sugar substitution. The fragmentation pathway of monoacetylated genistein derivatives started with elimination of a keten molecule ($CH_2$=C=O). Further fragmentation pathway followed retro Diels-Alder reaction. In the mass spectrum of 7-*O*-acetyl-4',5-di-*O*-methyl genistein fragment ions at 166 *m/z* (A ring) and 132 *m/z* (B ring) were observed, and for 4'-*O*-acetyl- isomer ions at 180 *m/z* and 118 *m/z* were registered (Fig. 6a and b). The retention time of both positional isomers differed by more than 1 minute. Also from the fragmentation pathway of 2'-hydroxygenistein derivative, obtained after chemical treatment of compound **3**, the site of sugar substitution was elucidated on hydroxyl at carbon C-7 of ring A. RDA reaction occurred

**Table 4. Aglycones and sugar identified after GC/MS analysis of methylated, methanolyzed and acetylated flavonoid-O glycosides 1-4**

| Compound | $t_R$ | MW | Flavonoid glycosides | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| 7-*O*-Acetyl-4',5-di-*O*-methylgenistein | 31.01 | 340 | + | - | - | - |
| 4'-*O*-Acetyl-5,7-di-*O*-methylgenistein | 31.91 | 340 | - | + | - | - |
| 2',4',5-Tri-*O*-methyl 2'-hydroxygenistein[a] | 33.24 | 328 | - | - | + | - |
| Tri-*O*-methyl-tetrahydroxyflavone[a] | 33.78 | 328 | - | - | - | + |
| 1,2,3,4,6-Penta-*O*-methyl-glucopyranoside | 4.05/4.64 | 250 | + | + | + | + |

[a]GC/MS analysis performed without acetylation; $t_R$, retention time.

in the C ring with proton transfer, and as a result fragment ions at 167 and 161 *m/z* were created (Fig. 6c). GC/MS analysis of compound **4**, because of small amounts of the sample, was not sufficient to give information about structure of the aglycone. It was only possible to identify 1,2,3,4,6-penta-*O*-methyl glucose in the reaction mixture after methylation and methanolysis (Table 4).

Because C-glucosides are resistant to acid hydrolysis, attempts at ferric chloride oxidation [25] and hydroiodic acid treatment [22] of compound **5** were performed in order to further identify the sugar and the aglycone. Good results were not achieved, and the reason for this was probably that too little sample (less than 1 mg) was available.

## CONCLUSION

The methodological approach presented above, where different mass spectrometric techniques and simple chemical modifications are used, enables the collection of complete structural information about investigated flavonoid O-glucosides **1–3**: genistein 7-*O*-glucoside **1**, genistein-4′-*O*-glucoside **2**, and 2′-hydroxygenistein-7-*O*-glucoside **3**, from submilligram quantities of samples. For the O-glucoside **4**, only molecular weight and identity of sugar were established. Except for molecular mass of aglycone and sugar gathered from desorption ionization mass spectra, it was possible to establish structures of the aglycone and the sugar ring after GC/MS analysis. In the latter case, comparison of retention times of permethylated sugars from the original samples with those of standards was necessary. Additionally, GC/MS performed on the acetylated derivatives allowed the estimation of the position of sugar substitution. From mass spectrometric data it was not possible to evaluate anomeric configuration of carbon C-1 in the sugar moiety. This information would be available after enzymatic hydrolysis of compounds with specific $\alpha$- or $\beta$-glucosidases.

For flavonoid C-glycoside **5**, its molecular weight was established. The aglycone moiety was recognized as trihydroxyflavone, (most probably apigenin), or isomeric isoflavone, and the substituted sugar was identified as a hexose. The position of sugar substitution was established after comparison of registered CID linked scan spectra and EI mass spectrum of permethylated derivative with literature data [13, 23].

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