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Fragmentation Pathways of Acylated Flavonoid Diglucuronides from Leaves of *Medicago truncatula*

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ABSTRACT:
Introduction – Flavonoids are important plant compounds occurring in tissues mostly in the form of glycoconjugates. Most frequently the sugar moiety is comprised of mono- or oligosaccharides consisting of common sugars like glucose, rhamnose or galactose. In some plant species the glycosidic moiety contains glucuronic acid and may be acylated by phenylpropenoic acids.
Methodology – Flavonoid glyconjugates were extracted from leaves of *Medicago truncatula* ecotype R108 and submitted to analysis using high-performance liquid chromatography combined with high-resolution tandem (quadrupole-time of flight, QToF) mass spectrometry.
Results – The studied leaf extracts contained 26 different flavonoid glycosides among which 22 compounds were flavone (apigenin, luteolin, chrysoeriol and tricin) glucuronides and 13 were acylated with aromatic acids (*p*-coumaric, ferulic or sinapic). The fragmentation pathways observed in positive and negative ion mass spectra differed substantially between each other and from these of flavonoid glycosides which did not contain acidic sugars. The application of high-resolution MS techniques allowed unequivocal differentiation between ions with the same nominal *m/z* values containing different substituents (e.g. ferulic acid or glucuronic acid). Eleven of the identified flavonoids have not been reported previously in this species.
Perspectives – The presented unique fragmentation pathways of flavonoid glucuronates enable detection of these compounds in tissue extracts from different plant species. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: Collision-induced dissociation; electrospray; flavonoid glucuronides; *Medicago truncatula*; tandem mass spectrometry

Introduction

Flavonoid glycosides constitute one of the largest groups of secondary metabolites synthesised by plants (Harborne and Williams, 2000; Andersen and Markham, 2006). This class of natural products plays an important role in plant physiology and biochemistry (Taylor and Grotewold, 2005), especially during interactions with other plants (allelopathy), pathogens (infection), herbivores, in the pollination processes or in response to abiotic stress factors like drought, light or temperature (Dixon and Paiva, 1995). Applications of liquid chromatography combined with mass spectrometry in studies of flavonoids are widely described in the literature and the use of different mass spectrometric analysers as well as ionisation methods has been reviewed (Stobiecki, 2000, 2001; Prasain *et al.*, 2004; Andersen and Markham, 2006; Stobiecki and Kachlicki 2006; Vacek *et al.*, 2008).

Flavonoid glucuronides form a particular group among the flavonoid glycosides. These compounds contain glucuronic acid moiety (or moieties) either instead or in addition to sugar units commonly present in plants. Flavonoid glucuronides occur in tissues of plants belonging to various taxa (Huang *et al.*, 1999; Kowalska *et al.*, 2007; Ringla *et al.*, 2007; Yamazaki *et al.*, 2007). On the other hand, flavonoid glucuronides are produced from flavonoids by UDP-glucuronosyltransferases (UGTs) as a result of the metabolism in animals and humans (Manach *et al.*, 2004). However, during these physiological processes most metabolites are singly glucuronidated on different hydroxyls of a flavonoid moiety, and LC-MS methods designed for analysis of flavonoid glucuronides in body fluids (blood, plasma and urine) deal mainly with such compounds (Mullen *et al.*, 2003; Davis *et al.*, 2006; Duenas *et al.*, 2008; Zhou *et al.* 2008).

Barrel medic (*Medicago truncatula* Gertn.) is closely related to alfalfa (*M. sativa* L.) and belongs to family Fabaceae along with

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other legume crop plants such as pea, soybean and lupins that are frequently used for foods and feeds. For this reason it became a convenient model plant for genetic research concerning the mentioned crops (Cook, 1999) and the number of studies on this species is increasing rapidly. As yet two lines of *M. truncatula*, namely Jemalong and R108, have been significantly improved for embryogenesis through cycles of *in vitro* culture to increase the regeneration efficiency and are commonly used in different research areas. It is worth noting that R108 may belong to a different *M. truncatula* subspecies (ssp. *tricycla*) to Jemalong (ssp. *truncatula*) (LeSignor *et al.*, 2005). A large number of metabolomic studies of this species regarding root tissues or cell cultures thereof have been conducted using different LC-MS techniques (Farag *et al.*, 2007, 2008). Twenty-three flavonoid glucosides, most of them glucuronides, have been isolated from aerial parts of *M. truncatula* plants and structurally characterised using NMR and MS methods by Kowalska *et al.* (2007). Among these compounds di- and triglucuronides of flavones apigenin (MW 270), luteolin (MW 286), chrysoeriol (MW 300) and tricin (MW 330) acylated with phenylpropenoic acids (ferulic, coumaric or sinapic) have been identified.

In our studies two LC-MS systems were used, aiming to elucidate the fragmentation pathways of *M. truncatula* flavonoids after collision-induced dissociation (CID) experiments. Both spectrometers were equipped with ESI ion sources and in one of them ion trap (IT) was used whereas the other had a high-resolution tandem quadrupole–time of flight (QToF) mass analyser. Distinct rules of charge retention for positive and negative ions on fragments created after bond cleavages could be demonstrated. Advantages of application of the high-resolution analyser and of registration of CID mass spectra in both negative and positive modes to draw structural conclusions about the analysed compounds are discussed.

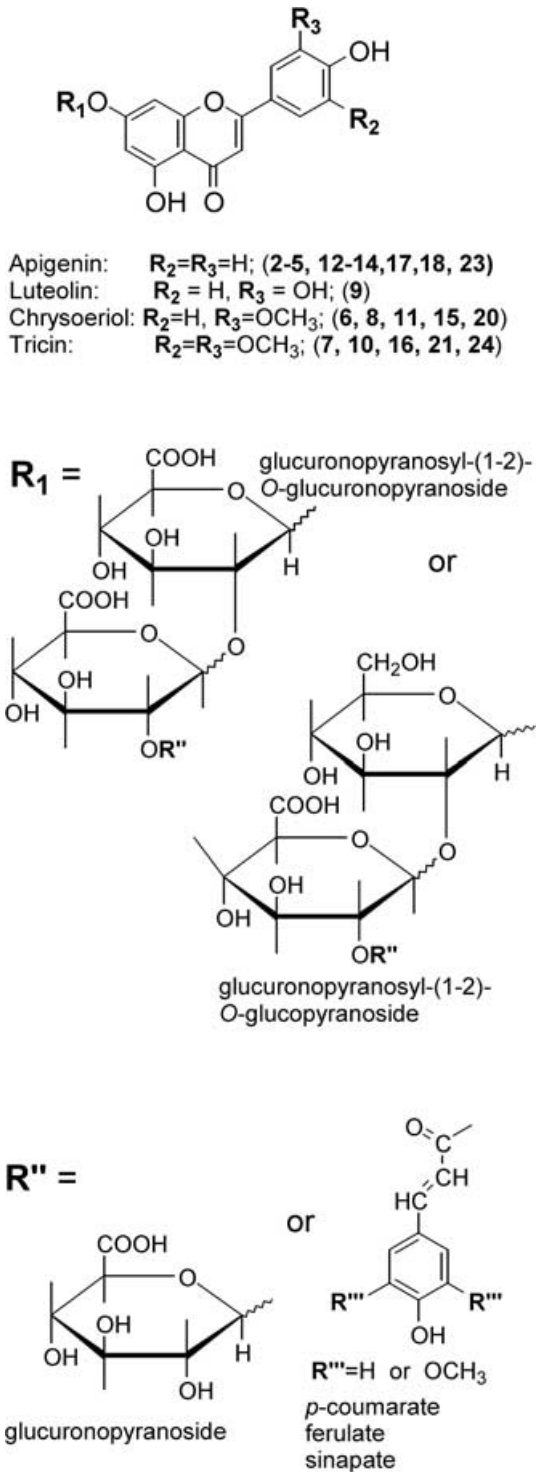

Figure 1. Structures of flavonoid glucuronides isolated from *M. truncatula* leaves.

Experimental

Reagents and standards

HPLC solvents were MS grade from Sigma (Poznań, Poland); ultra-pure water was obtained using a Millipore (Billerica, MA, USA) model MiliQ Plus system. Standards of flavonoids—chrysoeriol (3′-*O*-methylluteolin) and apigenin (structures shown in Fig. 1)—were from Extrasynthese (Genay, France).

Plant material

Plants (*Medicago truncatula* Gaertn. ecotype R108-1, seeds kindly provided by A. Kondorosi, CNRS, Gif-sur-Yvette, France) were grown in pots containing mixture of sand and perlite (1:1) under controlled greenhouse condition with an average temperature of 25°C, 50% humidity and a 16 h photoperiod. Plants were watered every 2 days and supplemented with fertiliser NPK (6:3:6, 3 g/L, 25 mL per pot) once a week. Six-week-old plants were used for the isolation of flavonoids.

Isolation of phenolic secondary metabolites from plant tissue

For the structural elucidation of flavonoid glucuronides, frozen plant material (2 g fresh weight) was homogenised in 80% methanol (12 mL), and the suspension was placed in an ultrasonic bath for 30 min. The extract was centrifuged and the supernatant was transferred to a new screw-cupped tube. Solvent was evaporated in a vacuum concentrator Savant SPD 121P (Thermo Electron Corporation, Waltham, MA, USA); during this procedure the tube was placed in a tray rotating in vacuum at room temperature. Dried extract samples were dissolved in water (3 mL) and subjected to solid-phase extraction (SPE) on silica gel RP C_{18} (400 mg) from Baker (USA). The loaded SPE columns were washed with 2 mL of 10% MeOH in H_2O and the adsorbed target natural products were eluted with 2 mL of methanol. The samples were immediately subjected to the LC-MS analyses.

Liquid chromatography with UV and mass spectrometric detection

Analyses of plant extracts were performed with two LC-UV-MS systems. The first of them consisted of an Agilent 1100 HPLC (Waldbronn, Germany) combined with an ion trap (IT) mass spectrometer, model Esquire 3000 (Bruker Daltonics, Bremen, Germany) and the second was an Agilent 1200 SL system (binary pump SL, diode array detector G1315C Starlight and automatic injector G1367C SL WP) connected to a micrOTOF-Q spectrometer from Bruker. In both instruments analyses were carried out using Zorbax Eclipse XDB-C_{18} columns (Agilent), differing in column size and granulation: 2.1 × 150 mm, grain diameter 3.5 μm and 2.1 × 100 mm, grain diameter 1.8 μm, respectively. Chromatographic runs at the Agilent 1200 system were performed at a 0.5 mL/min flow-rate using mixtures of two solvents: A (99.5% H_2O, 0.5% formic acid v/v) and B (99.5% acetonitrile, 0.5% formic acid, v/v) with a split of the column effluent of 3 : 2 so that 0.2 mL/min was delivered to the ESI ion source. The elution steps were as follows: 0–8 min linear gradient from 5 to 30% B, 8–10 min linear gradient to 95% B, 10–12 min isocratic at 95% B; after return to the initial conditions the equilibration was achieved after 3 min. The same solvents were used in the Agilent 1100 system at a flow rate of 0.2 mL/min and the gradient was: 0–35 min linear from 5 to 30% B, 35–45 min up to 95% B, 45–52 min isocratic at 95% B, return to initial conditions and re-equilibration (until 60 min).

The settings of the IT mass spectrometer were the following: electrospray ion source (ESI) voltage +4 or –4 kV (depending on the ion mode), nebulisation with nitrogen at 1.7 bar, dry gas flow 7 L/min, gas temperature 310°C, skimmer 1 voltage +12.4 or –11.2 V, collision energy set to 1 V and ramped within 40–200% of this value. The ion number accumulated in the trap was set to 10000 and the maximum accumulation time was 200 ms. According to the results of preliminary experiments, spectra were recorded in the targeted mode in the mass range *m/z* 50–1500. The instrument operated under EsquireControl version 5.1, and data were analysed using the DataAnalysis version 3.1 package delivered by Bruker.

The micrOTOF-Q spectrometer consisted of ESI operating at a voltage of ± 4.5 kV, nebulisation with nitrogen at 1.6 bar and dry gas flow of 8.0 L/min at a temperature of 220°C. The system was calibrated externally using a calibration mixture containing sodium formate clusters. Additional internal calibration was performed for every run by injection of the calibration mixture using the divert valve during the LC separation. All calculations were done with HPC quadratic algorithm. Such a calibration gave at least 5 ppm accuracy. MS/MS spectra were acquired with the frequency of one scan per second for ions chosen on the basis of preliminary experiments. Collision energy depended on molecular masses of compounds and was set between 10 and 25 eV. The instrument operated at a resolution higher than 10000 (FWHM, full width at half maximum) under the program micrOTOF Control version 2.3 and data was analysed using the DataAnalysis version 4 package delivered by Bruker. Metabolite profiles were registered in positive and negative ion modes. For identification of compounds the instrument operated in the targeted MS/MS mode and single ion chromatograms for exact masses of $[M + H]^+$ and $[M - H]^-$ ions (±0.005 Da) were recorded.

Results and Discussion

Flavonoid glycosides of *Medicago truncatula*

The LC-MS analyses of *M. truncatula* (ecotype R108-1) leaf extracts revealed the presence of at least 26 flavonoid glycosides for which complete sets of high-resolution MS/MS spectra as well as low-resolution MS^n spectra both in positive and negative ion modes were obtained. Most of the compounds detected were flavone glycosides being derivatives of apigenin, luteolin, chrysoeriol and tricin, containing glucose and/or glucuronic acid molecules (**2**–**24**, Table 1 and Fig. 1). Two compounds (**25**, **26**) were isoflavone formononetin and **1** was the flavonol laricitrin glucosides. Some of compounds **2**–**24** were acylated with ferulic or coumaric acid and one (**18**) with sinapic acid. The number of detected flavonoid conjugates from the ecotype R-108 was close to the 23 compounds previously identified in the ecotype Jemalong (Kowalska *et al.*, 2007). However, only 10 out of 26 compounds observed (**1**, **2**, **4**, **7**, **8**, **16**, **18**, **21**, **23**, **24**) were then described using different physicochemical methods (^1H and ^{13}C NMR, MS). In particular, phenylpropenoic acidic moieties (*p*-coumaric, ferulic and sinapic acids) of these conjugates were identified, positions of the ester bonds were established as well as those of glycosidic bonds between the aglycone and sugar or sugars (Kowalska *et al.*, 2007). In similar studies Stochmal *et al.* (2001a, b) showed that numerous flavonoid glucuronides were present in aerial parts of alfalfa (*M. sativa*) and in all those compounds the positions of ester and glycosidic bonds were identical to those from *M. truncatula*. On this basis it could be assumed that the enzymatic systems responsible for acylation and glucosidation of flavonoids had the same specificity in all these closely related species.

Complete sets of low resolution MS^n spectra of the detected compounds were recorded for their corresponding $[M + H]^+$ and $[M - H]^-$ ions. However, there is an important reason for which these spectra are not sufficient for an unambiguous identification of the protonated/deprotonated molecule ions as well as their CID fragmentation ions. Many different plant secondary metabolites occur in tissues in the form of glycosides acylated by phenylpropenoic acid derivatives such as *p*-coumaric, caffeic or ferulic acids. The main problem with the interpretation of mass spectra of these compounds arises from the fact that the neutral loss of these acyl groups (nominal masses of 146, 162 and 176, respectively) is equal to that observed for common glycosidic moieties (rhamnose, glucose or galactose and glucuronic acid). For this reason recording of high-resolution mass spectra is necessary for a proper identification of flavonoid conjugates containing these compounds or their derivatives. Metabolite profiling analyses in our experiments performed with the use of a ToF analyser in the LC-MS/MS system gave resolution higher than 10000 FWHM and it was possible to determine exact masses of protonated molecules $[M + H]^+$ and product ions, with a mass accuracy better than 5 ppm (Tables 1–3). The measurement accuracy and applied resolution permitted both classes of the analysed compounds to be distinguished. Single ion chromatograms of *m/z* values of $[M + H]^+$ precursor ions recorded for exact masses (±0.005 mass unit) permitted different compounds with the same nominal masses to be distinguished, for example compound **3** (calculated mass of the $[M + H]^+$ 785.1771) from **14** and **22** (calculated mass of the $[M + H]^+$ 785.1924) or compound **2** from **12** and **19** (calculated masses of respective $[M + H]^+$ ions: 799.1569 and 799.1716; Table 1). Additionally, this information along with the characteristic fragmentation pathways concluded from the positive and negative ion MS/MS spectra allowed a proper interpretation of the obtained data (Table 2 and 3). Another problem with the structural characterisation of the analysed compounds was connected to different patterns of methoxylation of flavonoid aglycones (apigenin, chrysoeriol and tricin) as well as of acyl groups (ferulic and *p*-coumaric acid) substituted on the sugar rings (Fig. 1). Finally, the chromatographic resolution was an important factor in the analysis due to the close structural similarities of some of the flavonoid glucuronides. It is noteworthy that the resolving power of the HPLC systems equipped with C_{18} columns with grains below 2 μm enabled the separation of compounds under study within 7 min

Table 1. Flavonoid glycosides identified in extracts from leaves of *M. truncatula* ecotype R108 plants

No.	R_t (min)	Compound	Exact isotopic mass of $[M + H]^+$ ions [calculated]	Exact isotopic mass of $[M + H]^+$ ions [observed]	Elemental composition
1	0.9	Laricitrin 3-*O*-glucopyranoside-5′-*O*-glucopyranosyl-7-*O*-glucoside[a]	819.2190	819.2223	$C_{34}H_{42}O_{23}$
2	1.9	Apigenin 7-*O*-glucuronopyranosyl-(1-3)-*O*-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside[a]	799.1569	799.1569	$C_{33}H_{34}O_{23}$
3	2.1	Apigenin 7-*O*-glucuronopyranosyl-(1-2)-*O*-glucuronopyranosyl-(1-2)-*O*-glucoside	785.1771	785.1776	$C_{33}H_{36}O_{22}$
4	2.2	Apigenin 7-*O*-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside[a]	623.1243	623.1263	$C_{27}H_{26}O_{17}$
5	2.4	Apigenin 7-*O*-glucuronopyranosyl-(1-2)-*O*-glucopyranoside	609.1451	609.1483	$C_{27}H_{28}O_{16}$
6	2.5	Chrysoeriol 7-*O*-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside	653.1348	653.1341	$C_{28}H_{28}O_{18}$
7	2.6	Tricin 7-*O*-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside[a]	683.1454	683.1438	$C_{29}H_{30}O_{19}$
8	2.8	Chrysoeriol 7-*O*-glucuronopyranosyl-(1-2)-*O*-glucopyranoside[a]	639.1556	639.1583	$C_{28}H_{30}O_{17}$
9	2.9	Luteolin 7-*O*- glucuronopyranoside	463.0872	463.0858	$C_{21}H_{18}O_{12}$
10	3.0	Tricin 7-*O*-glucuronopyranosyl-(1-2)-*O*-glucopyranoside	669.1662	669.1672	$C_{29}H_{32}O_{18}$
11	4.5	Chrysoeriol 7-*O*-glucopyranoside	463.1235	463.1250	$C_{22}H_{22}O_{11}$
12	4.7	Apigenin 7-*O*-[2′-*O*-feruloyl-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside]	799.1716	799.1747	$C_{37}H_{34}O_{20}$
13	4.8	Apigenin 7-*O*-[2′-*O*-coumaroyl-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside]	769.1611	769.1643	$C_{36}H_{32}O_{19}$
14	4.9	Apigenin 7-*O*-[2′-*O*-feruloyl-glucuronopyranosyl-(1-2)-*O*-glucopyranoside]	785.1924	785.1958	$C_{37}H_{36}O_{19}$
15	5.0	Chrysoeriol 7-*O*-[2′-*O*-feruloyl-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside]	829.1822	829.1827	$C_{38}H_{36}O_{21}$
16	5.0	tricin 7-*O*-[2′-*O*-feruloyl-Glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside][a]	859.1928	859.1975	$C_{39}H_{38}O_{22}$
17	5.0	Apigenin 7-*O*-[2′-*O*-coumaroyl-glucuronopyranosyl-(1-2)-*O*-glucopyranoside	755.1818	755.1845	$C_{36}H_{34}O_{18}$
18	5.1	Apigenin 7-*O*-[2′-*O*-sinapoyl-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside][a]	829.1822	829.1841	$C_{38}H_{36}O_{21}$
19	5.1	Chrysoeriol 7-*O*-[2′-*O*-coumaroyl-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside]	799.1716	799.1750	$C_{37}H_{34}O_{20}$
20	5.2	Chrysoeriol 7-*O*-[2′-*O*-feruloyl-glucuronopyranosyl-(1-2)-*O*-glucopyranoside]	815.2029	815.2030	$C_{38}H_{38}O_{20}$
21	5.3	Tricin 7-*O*-[2′-*O*-feruloyl-glucuronopyranosyl-(1-2)-*O*-glucopyranoside][a]	845.2135	845.2087	$C_{39}H_{40}O_{21}$
22	5.4	Chrysoeriol 7-*O*-[2′-*O*-coumaroyl-glucuronopyranosyl-(1-2)-*O*-glucopyranoside]	785.1924	785.1962	$C_{37}H_{36}O_{19}$
23	5.4	Apigenin 7-*O*-{2′-*O*-feruloyl-[glucuronopyranosyl-(1-3)]-*O*-glucuronopyranosyl-(1-2)-*O*glucuronopyranoside][a]	975.2037	975.2088	$C_{43}H_{42}O_{26}$
24	6.2	Tricin 7-*O*-[2′-*O*-coumaroyl-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside][a]	829.1822	829.1837	$C_{38}H_{36}O_{21}$
25	6.7	Formononetin 7-*O*-glucoside	431.1337	431.1351	$C_{22}H_{22}O_9$
26	7.3	Formononetin 7-*O*-glucoside malonylated	517.1341	517.1359	$C_{25}H_{24}O_{12}$

[a] Compounds identified previously (Kowalska *et al.*, 2007).

Table 2. Fragmentation pathways of acylated and non-acylated flavone glucuronides from *M. truncatula* observed in the positive ions mode

Compound (*m/z* of $[M + H]^+$)[a]	*M/z* of ions produced in result of different fragmentation pathways		
	Stepwise release of sugar (or acylated sugar) units to $\mathbf{Y_2}$, $\mathbf{Y_1}$ and $\mathbf{Y_0}$ ions[b]	Formation of ions $[M - aglycone - GlcA + H]^+$ Charge retention on acyl-sugar moiety[b]	Formation of ions $[M - aglycone - Glc + H]^+$ Charge retention on acyl-sugar moiety[b]
3 (785.1776)	609.1451, 433.1130, 271.0601	—	—
4 (623.1263)	447.0922, 271.0601	—	—
6 (653.1341)	477.1028, 301.0707	—	—
7 (683.1438)	507.1134, 331.0813	—	—
5 (609.1483)	433.1130, 271.0601	—	—
8 (639.1583)	463.1235, 301.0707	—	—
10 (669.1672)	493.1341, 331.0813	—	—
13 (769.1643)	447.0922, 271.0601	323.0762	—
19 (799.1750)	477.1028, 301.0707	323.0762	—
24 (829.1837)	507.1134, 331.0813	323.0762	—
12 (799.1747)	447.0922, 271.0601	353.0868	—
15 (829.1827)	477.1028, 301.0707	353.0868	—
16 (859.1975)	507.1134, 331.0813	353.0868	—
14 (785.1958)	433.1130, 271.0601	—	353.0868
20 (815.2030)	463.1235, 301.0707	—	353.0868
21 (845.2087)	493.1341, 331.0813	—	353.0868
17 (755.1845)	433.1130, 271.0601	—	323.0762
22 (785.1962)	463.1235, 301.0707	—	323.0762

[a] Observed *m/z* values for the respective ions.
[b] Calculated masses of ions.

Table 3. Fragmentation pathways of acylated and non-acylated flavone glucuronides from *M. truncatula* observed in the negative ions mode

Compound (*m/z* of $[M - H]^-$)[a]	*M/z* of ions produced in result of different fragmentation pathways				
	$[M - H]^-$-aglycone (further decomposition of saccharide or acyl saccharide)[b]	-saccharide or acyl saccharide to $\mathbf{Y_0}$ ions[b]	Breakdown of saccharide or acyl saccharide to $\mathbf{Y_1}$ and $\mathbf{Y_0}$ ions[b]	-phenylpropenoic diglucuronate to $\mathbf{Y_0}$ ions[b]	-ferulic acid to $\mathbf{Z_2}$ ions[b]
3 (783.1655)	513.1097; (351.0569)	—	—	—	—
4 (621.1092)	351.0569	269.0455	—	—	—
6 (651.1189)	351.0569	299.0561	—	—	—
7 (681.1323)	351.0569	329.0666	—	—	—
5 (607.1282)	—	—	431.0983, 269.0450	—	—
8 (637.1401)	—	—	461.1089, 299.0561	—	—
10 (667.1537)	—	—	491.1195, 329.0666	—	—
13 (767.1440)	497.0936 (333.0463)	—	—	269.0455	—
19 (797.1555)	497.0936 (333.0463)	—	—	299.0561	—
24 (827.1647)	497.0936 (333.0463)	—	—	329.0661	—
12 (797.1572)	527.1042 (333.0463)	—	—	269.0455	—
15 (827.1627)	527.1042 (333.0463)	—	—	299.0561	—
16 (857.1743)	527.1042 (333.0463)	—	—	329.0666	—
14 (783.1797)	513.1249	—	431.0983, 269.0455	—	589.1198
20 (813.1897)	513.1249	—	461.1089, 299.0561	—	619.1304
21 (843.1968)	513.1249	—	491.1195, 329.0666	—	649.1409
17 (753.1667)	483.1144	269.0455	—	—	—
22 (783.1757)	483.1144	299.0561	—	—	—

[a] Observed *m/z* values for the respective ions.
[b] Calculated masses of ions.

of a 15 min chromatographic run. Hyphenation of this system with a fast high-resolution mass spectrometer allowed the registration of MS/MS or pseudo-MS^3 spectra for consecutive compounds. For example, information necessary for the identification of nine compounds eluting in the 30 s time span between 4.7 and 5.2 min was achieved. It is noteworthy that the differences in natural contents of the studied compounds were higher than two orders of magnitude, according to abundances of the $[M + H]^+/[M - H]^-$ ions. Apigenin derivatives quantitatively dominated among the studied compounds: apigenin 7-*O*-glucuronopyranosyl-(1-2)-*O*-glucuronopyranosyl-(1-2)-*O*-glucoside, **3**; apigenin 7-*O*-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside, **4**; and apigenin 7-*O*-[2′-*O*-coumaroyl-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside], **12**.

Interesting differences were found between the profiles of flavonoid glycosides between R108 (this study) and Jemalong ecotypes (Kowalska *et al.*, 2007). Both ecotypes belong to different subspecies of *M. truncatula* (LeSignor *et al.*, 2005) and differ in several characteristics such as shape and pigmentation of leaves and flowers, symbiotic properties; some differences have also been revealed in their karyotypes (Cerbah *et al.*, 1999). Among 23 flavonoid glycosides previously identified in aerial parts of Jemalong plants (Kowalska *et al.*, 2007) there were 20 flavone derivatives and all of them contained glucuronic acid. Twenty-six compounds were presently identified in leaves of R-108 plants; 22 of them had glucuronic acid moiety (or moieties) and one (**11**) was a monoglucoside. Only one of the Jemalong compounds was a glucuronyl glucoside and seven such compounds were characterised in the R-108 ecotype. These differences support the diversity within the *M. truncatula* species. We cannot exclude the possibility that the differences might also arise from the conditions under which the plants were grown prior to the harvest, and from their stage of physiological development. In the present research plants were grown in a greenhouse, where part of the UV light was absorbed by the glass. Flavonoids are assumed to be responsible for protection of plant tissues against damaging UV-B radiation and acylation with aromatic acids substantially improves their capacity to absorb UV (Stochmal and Oleszek, 2007a,b). Therefore, it is possible that the intensity of acylation could be decreased under lower UV irradiation.

Fragmentation pathways of flavone di- and triglycosides

The flavone di- and triglycosides found in ecotype R108 of *M. truncatula* contained a linear saccharide attached at position 7 of apigenin, chrysoeriol and tricin which is crucial for the fragmentation behaviour of these compounds. The glycosidic moiety of compounds **3**, **4**, **6** and **7** consisted of two combined glucuronic acid molecules; that of compound **2** was a linear triglucuronate and **5**, **8**, **10** were glucuronosylo-glucosides. CID MS/MS spectra of all these compounds recorded in the positive mode contained only product ions created after successive cleavages of glycosidic bonds between sugars and between the sugar moiety and aglycone. The positive charge was retained on the aglycone (Table 2) and a whole series of Y_n^+ product ions was observed (Fig. 2, compound **3**). For the ion nomenclature see Domon and Costello (1988) and Claeys *et al.* (1996). This fragmentation pattern is identical to that observed for all flavonoid glycosides (Claeys *et al.*, 1996).

On the other hand, the pathway of the negative ions $[M - H]^-$ fragmentation strongly depended on the structure of the sugar moiety. Compounds containing two or three glucuronic acid molecules (**2**–**4**, **6**, **7**) yielded a charged glycosidic part of the molecule and a neutral flavone aglycone molecule, these product ions had a high abundance in all compounds (Fig. 2, Table 3). The diglucuronate moiety contained two carboxyl groups which strongly stabilised the product ions at *m/z* 351 (Fig. 3a) and for this reason the product ions bearing negative charge on the aglycone had very small intensities or were not registered at all. Fragmentation of the flavone glucuronosylo-glucosides (**5**, **8**, **10**) in the negative ion mode was similar to that observed in the positive ion mass spectra and product ions of Y_n type were created after cleavages of glycosidic bonds. Simultaneous elimination of both sugar rings resulting in a high abundance of Y_0 type ions occurred easily. Anyway, Y_1 ions were also recorded, so the sequence of sugar units could be revealed from these spectra. Generally, important structural information could be obtained from mass spectra of compounds **2**–**8**, **10**. The presence of the $[M - H - aglycone]^-$ product ions at *m/z* 527, 513 or 351 suggested, that all (two or three) sugar units were substituted at one hydroxyl group of the aglycone (most probably at C-7). It was possible to elucidate the sequence of sugars of apigenin triglycoside (**2**) and diglycosides (**5**, **8**, **10**) on the basis of *m/z* values of the product ions of Y_n type, registered in the positive ion CID MS/MS (Table 2) and consecutive MS^n spectra. It should be noted that the negative ion MS/MS spectra of various quercetin and methylquercetin diglucuronides isolated from animal tissue or body fluids were reported (Mullen *et al.*, 2003; Duenas *et al.*, 2008). For these compounds $[M - H]^-$ ions at *m/z* 653 or *m/z* 667 were observed and product ions were created after elimination of one or two sugars rings. However, no product ion at *m/z* 351 was then reported which might suggest that both sugars were attached to different hydroxyls on the aglycone.

Fragmentation pathways of acylated flavone diglycosides

Twelve compounds detected in leaves of plants from ecotype R108 of *M. truncatula* were diglycosides of flavones apigenin, chrysoeriol and tricin acylated mostly with either *p*-coumaric or ferulic acid. One compound (**18**, Table 1) was a sinapoyl derivative and one (**23**) was feruloyl triglucuronate. The fragmentation of the acylated flavonoid glycoconjugates (**12**–**24**) in the positive ion mode proceeded similarly to that of the non-acylated compounds (Table 2). Cleavages of glycosidic bonds between sugars and between the sugar and aglycone with the charge retention on the ions containing flavone moiety were the most frequent. However, the positive charge could be also found on the fragment composed of an acyl group (*p*-coumaroyl or feruloyl) and glucuronic acid molecule. These ions at *m/z* 323 (observed for compounds **13**, **17**, **19**, **22**, **24**) or *m/z* 353 (**12**, **14**–**16**, **20**, **21**), respectively, were created as a result of cleavage of the glycosidic bond between both sugars (Figs. 2, 3b and 4; Table 2). Only the registration of ion masses with a high accuracy permitted flavone triglycosides (e.g. **3**) to be distinguished from flavone diglucuronides acylated with ferulic acid (e.g. **14**). The monoisotopic exact mass of the $[M + H]^+$ ion at *m/z* 785 for elemental composition $C_{33}H_{36}O_{22}$ (compound **3**) was 785.1771 amu and for $C_{37}H_{36}O_{19}$ (compounds **14** and **22**) was 785.1924 (19 ppm difference). As the accuracy of *m/z* values registered for consecutive ions was better than 5 ppm these ions could be easily differentiated. Similar calculations conducted for the product ions at *m/z* 323.0762 and 353.0868 could easily allow them

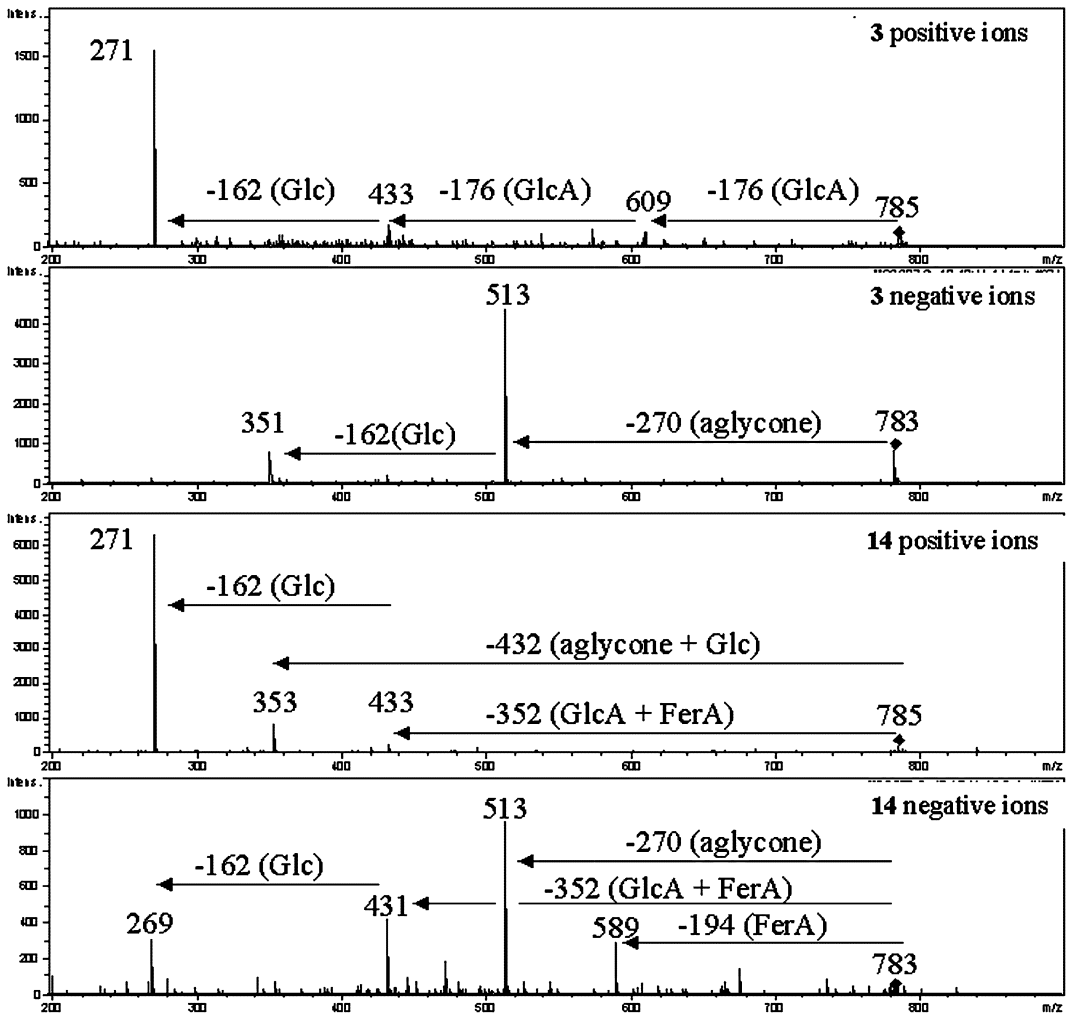

Figure 2. Mass spectra registered in positive and negative mode for: apigenin 7-*O*-glucuronopyranosyl-(1-2)-*O*-glucuronopyranosyl-(1-2)-*O*-glucoside, MW = 784 (**3**) and apigenin 7-*O*-[2′-*O*-feruloyl-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside] MW = 784 (**14**).

to be assigned to *p*-coumaroyl- and feruloyl-glucuronic acid, respectively.

The fragmentation of acylated glucuronyl glycosides of flavones in the negative ion mode was, similarly to the nonacylated compounds, different from that observed in the positive ions. The first step of this process for the $[M - H]^-$ ions of these compounds yielded two types of ions: abundant $[M - H - \text{aglycone}]^-$ ions and much less intensive $[\text{aglycone} - H]^-$ ions (Fig. 4; Table 3). The elimination of the aglycone resulted in the appearance of ions at *m/z* 527 and *m/z* 497 for compounds containing ferulic and *p*-coumaric acid, respectively (Fig. 4), from which the acyl was lost in the next step, giving the product ion at *m/z* 333 (Table 3; Figs. 3c and 4). This sequence of fragmentation could be revealed using the IT instrument giving sequential MS^n spectra. A little different mechanism was observed in the fragmentation of $[M - H]^-$ ions of acylated glucuronyl-glucosides (**14**, **17** and **20**–**22**). Similarly to the acylated diglucuronides, the most intensive fragmentation pathway released a neutral aglycone molecule, giving the product ions at *m/z* 513 or *m/z* 483 (compound **14** at Figs. 2 and 3d; Table 3) and additionally small amounts of the $[\text{aglycone} - H]^-$ ions were also detected. However, a remarkable difference between the fragmentation mechanism of feruloyl and *p*-coumaroyl compounds was seen. The elimination of ferulic acid molecule from compounds **14**, **20** and **21**) was observed (Fig. 2, Table 3) which caused creation of *Z* type fragment ions at *m/z* 589, 619 and 649, whereas no acyl loss could be seen for *p*-coumaroyl derivatives **17** and **22**. The fragmentation pathway for coumaroyl derivatives resembled that observed for diglucuronides **12**, **13**, **15**, **16**, **19**, **20** and **24** (Table 3).

The use of an LC-MS/MS system consisting of a rapid resolution HPLC chromatograph and a tandem MS/MS spectrometer equipped with a high-resolution time of flight analyser permits profound structural characterisation of flavonoid glycoconjugates containing glucuronic acid and aromatic acyl substituents. However, due to complementary information resulting from differences in fragmentation mechanisms of these compounds observed for their positive and negative ions, it is essential to perform analyses in both modes of ionisation. It is of particular importance that, especially in the negative ion mode, mechanisms observed for glycosides containing more than one molecule of glucuronic acid are dramatically different from those of

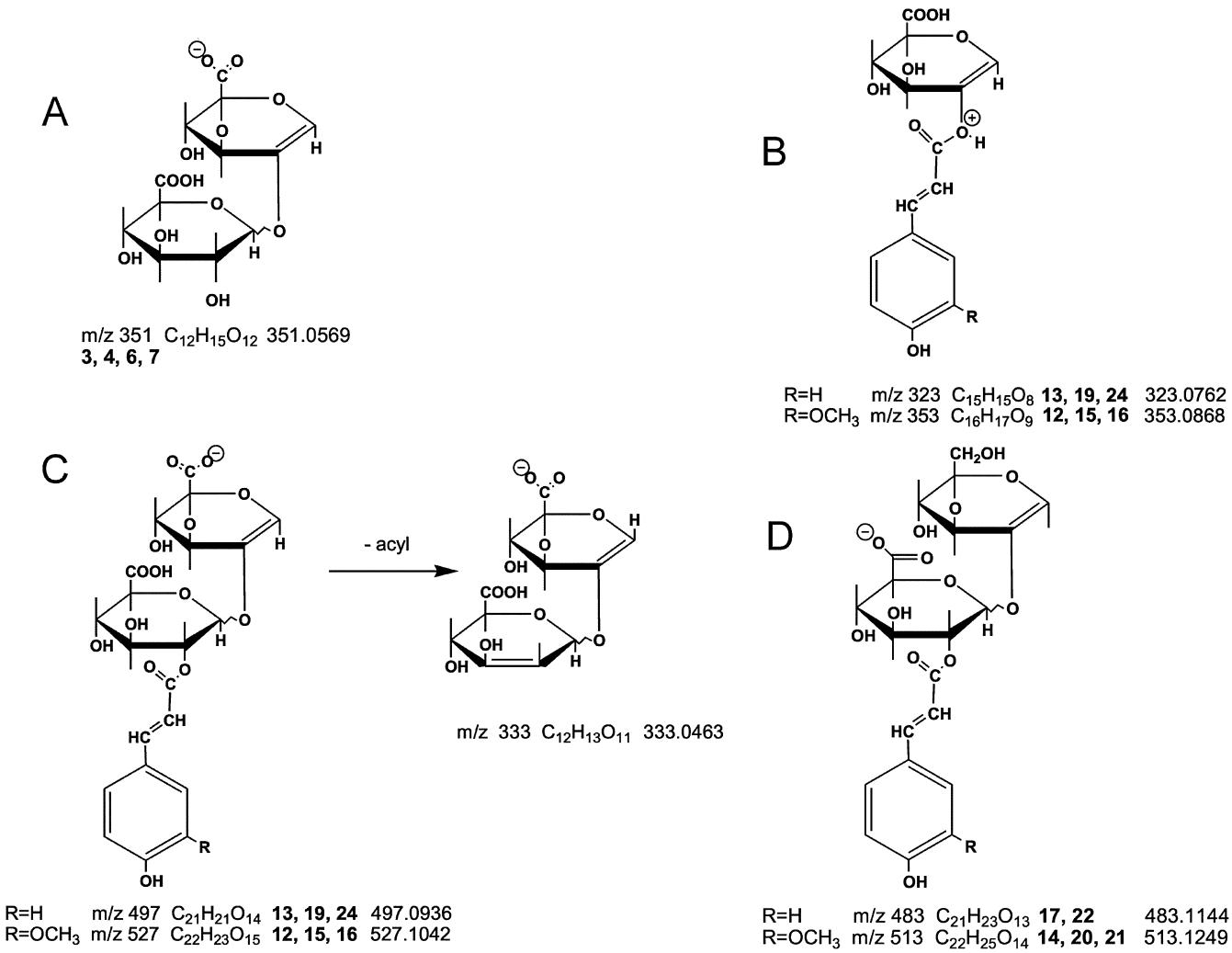

Figure 3. Structures of the most characteristic product ions of flavone glucuronates registered in the CID MS/MS spectra and their nominal and exact *m/z*: (A) product ion for compounds **3**, **4**, **6**, **7** in negative ions mode; (B) product ions for compounds **12**, **13**, **15**, **16**, **19**, **24** in positive ions mode; (C) product ions for compounds **12**, **13**, **15**, **16**, **19**, **24** in negative ions mode; (D) product ions for compounds **14**, **17**, **20–22** in negative ions mode.

flavonoid glycoconjugates containing sugars without carboxyl groups. The well-established fragmentation scheme of flavonoid glycosides is the stepwise release of sugar units with the eventual appearance of $[aglycone - H]^-$ ions (Claeys *et al.*, 1996) and their further breakdown. Such an mechanism of fragmentation may be in some cases observed for diglucuronides, but the appearance of $[M - H - aglycone]^-$ ions is the major pathway which gives a characteristic ion at *m/z* 351 (Table 3). Additionally, the acylation of these molecules with phenylpropenoic acids (ferulic, *p*-coumaric) influences substantially the fragmentation pathways. The CID fragmentation of these compounds yields several ions in which charge is retained on the phenylpropenoyl-glucuronate moiety (Tables 2 and 3; Fig. 3). Nevertheless, HR-MS analyses performed both in positive and negative ion modes may permit a proper structural characterisation of glucuronyloglucosides and di- or triglucuronides and enable to distinguish them from compounds in which a feruloyl group is present. Ions described in Table 2 and 3 may be used as diagnostic ones for a correct recognition of respective compounds according to the CID MS/MS experiments. Because of large differences in concentrations of the natural products in the analysed samples, monitoring of all compounds of interest present in *M. truncatula* leaves is possible only in the targeted mode of analysis. The application of a low-resolution mass spectrometer such as the IT instrument also used in our experiments does not always allow proper identification of all compounds. Because of equal nominal masses of different substituents such as glucuronic and ferulic acids, these derivatives may be correctly analysed only using a high-resolution instrument. On the other hand, decent fragmentations of some minor compounds present in the analysed samples have been obtained only using the IT instrument.

Acknowledgement

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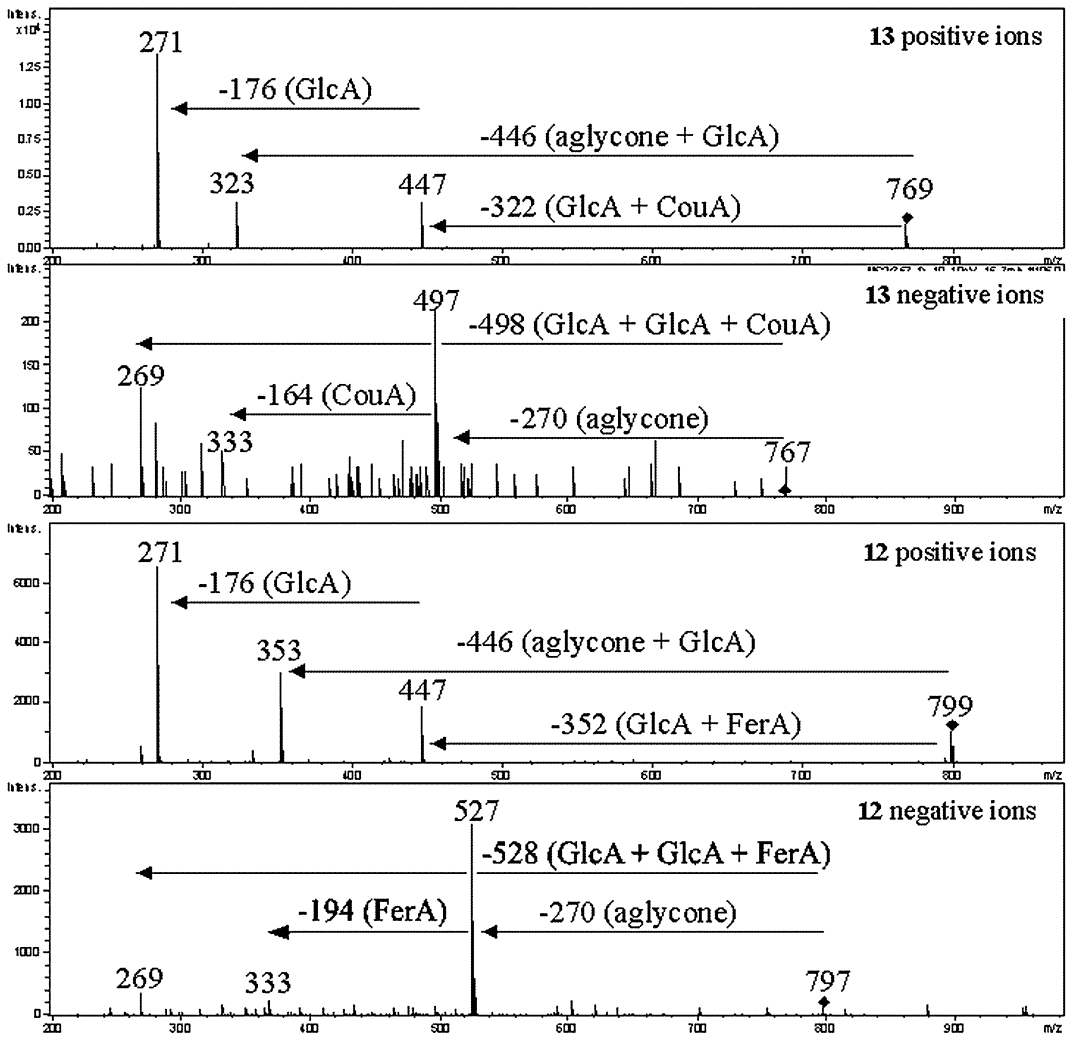

Figure 4. Mass spectra registered in positive and negative mode for: apigenin 7-*O*-[2′-*O*-feuroyl-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside], MW = 798 (**12**) and apigenin 7-*O*-[2′-*O*-coumaroyl-glucuronopyranosyl-(1-2)-*O*-glucuronopyranoside], MW = 768 (**13**).

References

Andersen ØM, Markham KR. 2006. *Flavonoids: Chemistry, Biochemistry and Applications*. CRC Press: Boca Raton, FL.

Cerbah M, Kevei Z, Siljak-Yakovlev S, Kondorosi E, Kondorosi A, Trinh TH. 1999. FISH chromosome mapping allowing karyotype analysis in *Medicago truncatula* lines Jemalong J5 and R-108-1. *Mol Plant Microbe Int* **12**: 947–950.

Claeys M, Li Q, van den Heuvel H, Dillen L. 1996. Mass spectrometric studies on flavonoid glucosides. In: *Applications of Modern Mass Spectrometry in Plant Sciences*, Newton RP and Walton TJ. (eds) Clarendon Press: Oxford; 182–194.

Cook D. 1999. *Medicago truncatula*—a model in the making! *Curr Opin Plant Biol* **2**: 301–304.

Davis BD, Needs PW, Kroon PA, Brodbelt JS. 2006. Identification of isomeric flavonoid glucuronides in urine and plasma by metal complexation and LC-ESI-MS/MS. *J Mass Spectrom* **41**: 911–920.

Dixon RA, Paiva NL. 1995. Stress-induced phenylpropanoid metabolism. *Plant Cell* **7**: 1085–1097.

Domon B, Costello CE. 1988. A systematic nomenclature for carbohydrate fragmentations in FAB MS/MS spectra of glycoconjugates. *Glycoconjug J* **5**: 397–409.

Duenas M, Mingo-Chornet H, Perez-Alonso JJ, Di Paola-Naranjo R, Gonzalez-Paramas AM, Santos-Buelga C. 2008. Preparation of quercetin glucuronides and characterization by HPLC-DAD-ESI/MS. *Eur Food Res Technol* **227**: 1069–1076.

Farag MA, Huhman DV, Lei Z, Sumner LW. 2007. Metabolic profiling and systematic identification of flavonoids and isoflavonoids in roots and cell suspension cultures of *Medicago truncatula* using HPLC-UV-ESI-MS and GC-MS. *Phytochemistry* **68**: 342–354.

Farag MA, Huhman DV, Dixon RA, Sumner LW. 2008. Metabolomics reveals novel pathways and differential mechanistic and elicitor-specific responses in phenylpropanoid and isoflavonoid biosynthesis in *Medicago truncatula* cell cultures. *Plant Physiol* **146**: 387–402.

Harborne JB, Williams CA. 2000. Advances in flavonoid research since 1992. *Phytochemistry* **55**: 481–504.

Huang Y, De Bruyne T, Apers S, Ma YL, Claeys M, Pieters L, Vlietinck A. 1999. Flavonoid glucuronides from *Picria fel-terrae*. *Phytochemistry* **52**: 1701–1703.

Kowalska I, Stochmal A, Kapusta I, Janda B, Pizza C, Piacente S, Oleszek W. 2007. Flavonoids from barrel medic (*Medicago truncatula*) aerial parts. *J. Agric. Food Chem.* **55**: 2645–2652.

Le Signor C, Gallardo K, Prosperi JM, Salon C, Quillien L, Thompson R, Duc G. 2005. Genetic diversity for seed protein composition in *Medicago truncatula*. *Plant Gen Resourc* **3**: 59–71.

Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. 2004. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **79**: 727–747.

Mullen W, Hartley RC, Crozier A. 2003. Detection and identification of 14C-labelled flavonol metabolites by high performance liquid chromatography-radiocounting and tandem mass spectrometry. *J Chromatogr A* **1007**: 21–29.

Prasain JK, Wang CC, Barnes S. 2004. Mass spectrometric methods for the determination of flavonoids in biological samples. *Free Radicals Biol Med* **37**: 1324–1350.

Ringla A, Prinza S, Huefnerb A, Kurzmannc M, Kopp B. 2007. Chemosystematic value of flavonoids from *Crataegus*×*macrocarpa* (Rosaceae) with special emphasis on (*R*)- and (*S*)-eriodictyol-7-*O*-glucuronide and luteolin-7-*O*-glucuronide. *Chem Biodivers* **4**: 154–162.

Stobiecki M. 2000. Review—application of mass spectrometry for identification and structural studies of flavonoid glycosides. *Phytochemistry* **54**: 237–256.

Stobiecki M. 2001. Applications of separation techniques hyphenated to mass spectrometer for metabolic profiling. *Curr Opin Chem* **5**: 89–111.

Stobiecki M, Kachlicki P. 2006. Isolation and identification of flavonoids. In: *The Science of Flavonoids*, Grotevold E (ed.). Springer Science and Business Media: New York; 47–69.

Stochmal A, Oleszek W. 2007a. Seasonal and structural changes of flavones in alfalfa (*Medicago sativa*) aerial parts. *J Food Agric Environ* **5**: 170–174.

Stochmal A, Oleszek W. 2007b. Effect of acylation of flavones with hydroxycinnamic acids on their spectral characteristics. *Nat Prod Commun* **2**: 571–574.

Stochmal A, Piacente S, Pizza C, De Riccardis F, Leitz R, Oleszek W. 2001a. Alfalfa (*Medicago sativa* L.) flavonoids. 1. Apigenin and luteolin glycosides from aerial parts. *J Agric Food Chem* **49**: 753–758.

Stochmal A, Simonet AM, Macias FA, Oleszek W. 2001b. Alfalfa (*Medicago sativa* L.) flavonoids. 2. Tricin and chrysoeriol glycosides from aerial parts. *J Agric Food Chem*. **49**: 5310–5314.

Taylor LP, Grotewold E. 2005. Flavonoids as developmental regulators. *Curr Opin Plant Biol* **8**: 317–323.

Vacek J, Klejdus B, Lojkova L, Kuban V. 2008. Current trends in isolation, separation, determination and identification of isoflavones: a review. *J Sep Sci* **31**: 2054–2067.

Yamazaki K, Iwashina T, Kitajima J, Gamou Y, Yoshida A, Tannowa T. 2007. External and internal flavonoids from *Madagascarian uncarina* species (Pedaliaceae). *Biochem Syst Ecol* **35**: 743–749.

Zhou DY, Xing R, Xu Q, Xue XY, Zhang FF, Liang XM. 2008. Polymethoxylated flavones metabolites in rat plasma after the consumption of *Fructus aurantii* extract: analysis by liquid chromatography/electrospray ion trap mass spectrometry. *J Pharm Biomed Anal* **46**: 543–549.